Recombinant Microtubule End-Capping Proteins As Inorganic Surface Ligands

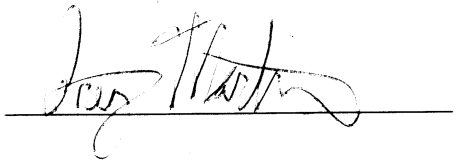
A Master Thesis Written By:
Greg Martin

Under the Supervision and Guidance Of:
Dr. Pierre Deymier

Statement by Author

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: 

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Dr. Pierre Deymier

04/07/2007

Date

Table of Contents

List of Figures - I

Abstract - II

Microtubules are being investigated as organic templates for nanowire fabrication. Targeted immobilization of the plus and minus ends of the microtubule can be achieved using recombinant end tracking microtubule proteins. This work examines the effects of five recombinant microtubule proteins on microtubule polymerization to determine their usefulness as inorganic surface ligands. GST-GAMMA-tubulin was found to retain its microtubule nucleating capacity and anchor microtubules to an inorganic surface. GST-CLIP-170-H2, HIS-CLIP-170-H2, GST-P150glued, and GST-EB1 were all found to retain their microtubule binding capacity. GST-P150glued and GST-EB1 were used to immobilize microtubules to inorganic surfaces. Their activity was enhanced by the presence of the H2 fragments.

Introduction - III

A. Microtubules As Nanowire Templates

In vivo, mictroubules are cellular structures found in the cytoplasm of eukaryotic cells. They are involved in many cellular processes including motility, cell division, and transport. Microtubules are protein filaments composed of tubulin subunits. Alpha and beta tubulin monomers associate with one another to form a heterodimer in solution.

Each monomer is approximately 450 amino acids and has a molecular mass of 50,000Da (1). The tubulin heterodimer is about 8nm long and has a diameter of 4-5nm (2). Tubulin heterodimers stack end-to-end to form protofilaments. A microtubule is composed of a tube of thirteen protofilaments arranged into a helical lattice. Due to the structural differences between α and β tubulin, microtubules are polarized structures with unique properties corresponding to each end.

Microtubules are only 24 nanometers in diameter but can grow up to several microns in length. This relatively large geometric aspect ratio has made microtubules an attractive candidate for use as nanowire templates. Their ability to self-assemble from aqueous solutions of protein subunits by simply raising the temperature of the reaction vessel makes low-cost batch fabrication of entire circuits a possibility. A method for metallizing microtubules by electroless deposition has already been described, (2,3) and characterized by electron microscopy (4). Parallel assembly of complex circuits could be accomplished without any manual manipulation if adequate control over the targeting of nucleation and capture could be achieved.

In vivo, the microtubule is accompanied by a vast array of microtubule-associated proteins (MAPs). These are proteins that

interact with both microtubules and each other to regulate microtubule polymerization, localization, and function. Complexes of these proteins are known to form structures called caps at the tips of microtubules. Caps prevent disassembly and participate in anchoring microtubules to other structures. The high specificity of these biological interactions can be utilized to achieve exquisite control over the fabrication process at the nanometer scale.

Dividing cells are able to direct the polymerization of microtubules between centrosomes and kinetochores with near-perfect accuracy during anaphase. These structures represent target areas of about $0.5\mu m^2$ each. *In vitro* this search-and-capture strategy could be mimicked by exposing end-anchored microtubules to cycles of heating and cooling in solution of free tubulin dimer. This would induce growth and collapse of microtubules at random vectors from the anchoring site. Upon capture of the dynamic end, the microtubule would be stabilized and protected against depolymerization pending metallization.

Surface adhesion complexes incorporating recombinant end-capping proteins would allow for the targeted anchoring of microtubule nanowire templates to metal electrodes. Recombinant versions of several end-capping MAPs have already been engineered into plus and minus end specific surface adhesion complexes. Eventually, a library of recombinant proteins could provide enough multiplicity between paired target sites to construct complex circuits.

B. Microtubule Polymerization

The proximal end exposing α-tubulin has been termed the minus end. This is the end of the microtubule where nucleation occurs *in vivo*. The mechanisms and structures involved in microtubule nucleation are not completely understood at the present.

Consolidation of various models describing spontaneous homogenous nucleation, heterogenous microtubule nucleation, and microtubule dynamic instability into a unified theory of microtubule polymerization is an ongoing effort. The characterization of various microtubule associated proteins (MAPs), drugs, and other small molecules involved in nucleation has revealed an extremely complex system of signaling and regulation. This work focuses on qualitative evaluations of microtubule nucleation and polymerization dynamics.

The distal end of the microtubule exposes a β-tubulin subunit and has been termed the plus end. While both ends of the microtubule are capable of the addition and removal of tubulin heterodimers, the plus end is 5-10 times more dynamically active than the minus end (5). *In vivo* during eukaryotic cell division, the plus end is the site of microtubule capture and subsequent stabilization. Growth of astral microtubules is directed towards the kinetochore of dividing chromosomes. When the plus end of a mitotic microtubule properly encounters a kinetochore, it is anchored and capped preventing subsequent depolymerization.

Each α and β tubulin monomer binds to one molecule of guanosine triphosphate (GTP) to form Tu-GTP. The GTP bound to β-tubulin is exchangeable, while the GTP bound to α- tubulin is non-exchangeable. The addition of a α/β heterodimer to the end of a growing microtubule is effectively coupled to the hydrolysis of the β-tubulin bound GTP. The resulting Tu-GDP is non-exchangeable. Hydrolysis of GTP changes the conformation of tubulin from curved to straight enhancing the lateral presentation of tubulin dimers to one another (6).

Prior to hydrolysis, the region of the microtubule plus end comprising the unhydrolyzed layer of Tu-GTP is termed the cap. The details regarding its structure and mechanism are still quite unclear.

Presently, the predominant model describes a lateral cap composed of a single layer of Tu-GTP subunits. The hydrolysis of the exposed Tu-GTP to Tu-GDP is effectively coupled to the addition of a replacement Tu-GTP dimer to the cap (7,8). The presence of this cap is thought to stabilize the microtubule and prevent its collapse.

Hydrolysis of the plus end Tu-GTP taking place without the subsequent addition of a replacement heterodimer, presumably due to a lack of free Tu-GTP dimers in solution, results in a destabilization of the plus end of the microtubule. The resulting event is called catastrophe and represents a rapid and significant collapse of the microtubule from the distal end due to dissociation of uncapped Tu-GDP. When a catastrophe, or rapid disassembly event, is followed by a growth phase, this is called a rescue event. Presumable this results from the addition of a new layer of Tu-GTP and a replacement of the plus end cap.

Dynamic instability is the term used to describe the exchange of free tubulin dimer with constitutive microtubule dimer via cycling between phases of slow growth or rescue, pausing, and catastrophe. The alternative process that describes the dynamic behavior of microtubules at a steady state length is treadmilling. This refers to the migration of individual tubulin subunits along the microtubule itself, from the distal plus end to the proximal minus end. This process can occur with both ends anchored and like dynamic instability, requires a constant supply of GTP (1).

In the presence of GTP and heat, α/β tubulin dimers will self-assemble into microtubules, provided that the free dimer concentration is greater than the critical concentration. The critical concentration is the minimum concentration required for microtubule polymerization

and dynamic instability to occur. Under standard conditions and without the presence of drug the observed critical concentration of tubulin is .2mg/mL (1.8μM) (9,10).

This value is dynamic and can be raised or lowered in the presence of small molecules, drugs, and proteins that interact with microtubules to stabilize them and promote assembly or destabilize them and promote disassembly. These factors can alter the polymerization kinetics of the microtubules and/or affect the stability of already formed microtubules. Taxol (paclitaxel) is an example of a drug that stabilizes microtubules and promotes assembly by lowering the critical concentration (9). Taxol binds to polymerized microtubules at an exposed site on β- tubulin with a stoichiometry of 1:1 (11). The binding of taxol to microtubules induces conformational changes that prevent the catastrophe that would normally result from the loss of the plus end cap. In the presence of as little as 5μM Taxol the critical concentration drops to ≤0.01mg/mL (9). In addition to altering the microtubule polymerization kinetics, Taxol can be used to stabilize preformed microtubules. A solution containing 10uM Taxol is capable of maintaining microtubules for up to a week.

The effect of some MAPs on microtubule polymerization dynamics can be similar to that of a drug like Taxol, depending on what the normal function of the protein in question is. Changes in the critical concentration, dissociation rates, growth rates, length distributions, and other polymerization parameters of microtubules polymerized in solution can be used as markers to evaluate whether a recombinant MAP has retained its interaction with microtubules.

Minus End Nucleation *In Situ* - IV

A. Microtubule Nucleation

Microtubule nucleation is the process in which free tubulin dimers assemble and associate into a microtubule seed. This seed acts as a template for the addition and removal of polymerizing tubulin dimers. *In vitro,* nucleation will occur spontaneously in solutions of free tubulin, provided that the concentrations of tubulin dimer and GTP are high enough. This happens at a rate that is dependent on the initial concentrations of GTP and tubulin. While the exact mechanism is unknown, the formation of two-dimensional sheets of tubulin has been well documented in solutions of purified tubulin (12). It is thought that these sheets fold into rings that act as nucleating seeds by forming templates for the addition of GTP bound tubulin heterodimers.

In vivo nucleation occurs at microtubule organizing centers. During mitosis in dividing cells the centrosome acts as a microtubule organizing center (MTOC) to regulate nucleation. Large protein complexes called gamma tubulin ring complexes (γ-TuRCs) are the site of nucleation. Gamma tubulin is a monomeric species of tubulin that is structurally similar to beta tubulin (13). It's presence is required for the nucleation of microtubules *in vivo*(6,13).

The structure of gamma tubulin is such that in the unbound state it's conformation allows for lateral interactions between monomers(6). The formation of γ-TuRCs is not a requisite for nucleation, however. Monomeric γ-tubulin has been show to nucleate microtubule growth *in vitro* (13). As a monomer, γ-tubulin lowers the size of the nucleus from seven to three tubulin subunits (13) by enhancing the intrinsically weak lateral interactions between dimers (6). Oligomers of γ-tubulin in γ-TuRCs act as a template for nucleation (14). The

binding of gamma tubulin to the minus end of a microtubule caps the minus end, preventing growth and polymerization (13,15). The nucleating mechanism of gamma tubulin as well as its exact interactions with microtubules and tubulin is still not completely understood (16).

We have engineered a recombinant version of gamma tubulin that has been tagged with glutathione-S-transferase. This GST-γ-tubulin will bind to a surface adhesion complex containing an antibody to GST. Microtubules nucleated from this GST-γ-tubulin will therefore be anchored at their minus end, assuming that the recombinant protein is viable. In this work the nucleating capacity of GST-γ-tubulin has been evaluated in solution with polymerizing microtubules and from surfaces functionalized with it as part of an adhesion complex.

B. Effects of GST-γ-Tubulin *In Situ*

The presence of nucleating seeds in solution has predictable effects on the polymerization dynamics of microtubules. Spontaneous nucleation of free tubulin dimer is slow without a nucleating agent. This implies a lag time between the initiation of polymerization and the appearance of microtubules of about 12 minutes under standard conditions (13). The addition of γ-tubulin reduces this observed lag time in a dose dependent manner (13).

When we polymerized purified tubulin in the presence of GST-γ-tubulin, long microtubules (>5μm) were observed after 10 minutes (fig. 1). Length distributions were obtained by measuring the microtubules from at least three different images per trial, and three different trials per treatment group. All of the counts for each treatment group were summed together. Images were selected to be representative samples of each group.

(a)

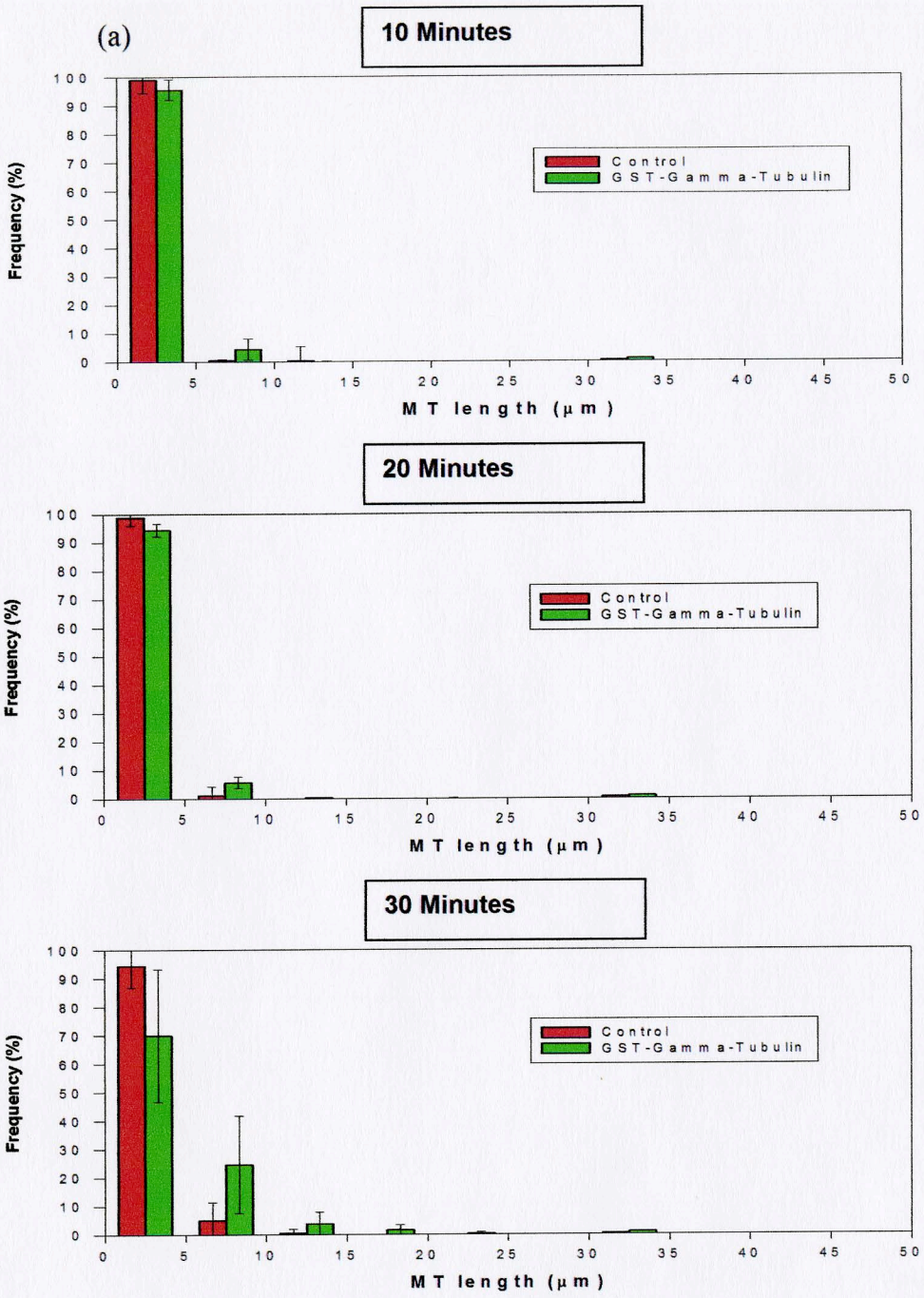
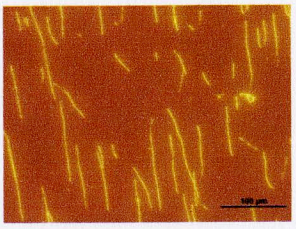


Figure 1 – (a) The three figures above show length distributions generated when 0.17mg/mL Purified Rhodamine-tubulin was polymerized at 37°C in PEM buffer (80mM Na-PIPES pH6.9, 1mM MgCl2, 1mM EGTA), 0.21mM GTP, and 16.7uM Taxol alone (A) and with 0.008mg/mL GST-γ-Tubulin (B) for 10, 20, and 30 minutes. The presence of microtubules >5μm after ten minutes in the GST-gamma-tubulin treated groups indicates an attenuation of the nucleation lag phase due to the nucleating effects of the recombinant protein. After 30 minutes the GST-γ-Tubulin groups contained longer microtubules. This supports a longer growth phase and minus end stabilization brought on by the nucleating activity of the GST-gamma-tubulin construct. (b) The image to the right is an example of the images that were used to generate length distributions.

(b)



A stoichiometric evaluation of the solution conditions represented by figure one suggests that only a small fraction of the total GST-γ-tubulin present is functioning as a minus end cap. The concentrations of tubulin dimer and GST-γ-tubulin were 1.7nM(0.17mg/mL) and 0.16nM respectively. Assuming that a microtubule is composed of thirteen tubulins arranged in a ring, this ratio corresponds to seventeen rows of tubulin dimer per nucleating cap of GST-γ-tubulin. This also assumes that thirteen gamma tubulin are arranged into a minus end cap at a ratio of one gamma tubulin to each tubulin dimer. As we mentioned earlier, each tubulin dimer has a length of 8nm, with about 1650 dimers constituting one micron of microtubule. If 100% of the GST-γ-tubulin in solution was active as a nucleator and bound to the minus end of a microtubule, we would expect to see the microtubules grow to a maximum length of 0.136μm before depleting all of the free tubulin from the solution. The distribution we observed for these conditions indicated an average length closer to ~5μm. To reach these lengths with a 17:1 ratio of tubulin dimer to GST-γ-tubulin, only about 2.5% of the available GST-γ-tubulin could have been bound to tubulin dimer. Such a low viability would probably be unacceptable for a surface ligand considering this statistic alone. Another explanation for this analysis is the presence of many undeveloped nucleation sites below the detection limit. These may consist of a ring of GST-γ-tubulin bound to a small number of tubulin dimers. Without affecting the length distribution, these nuclei could sequester most of the GST-γ-tubulin without significantly altering the free tubulin concentration.

In a computer simulation of microtubule seeding where various amounts of preformed seeds were added to a polymerizing solution, the

(a)

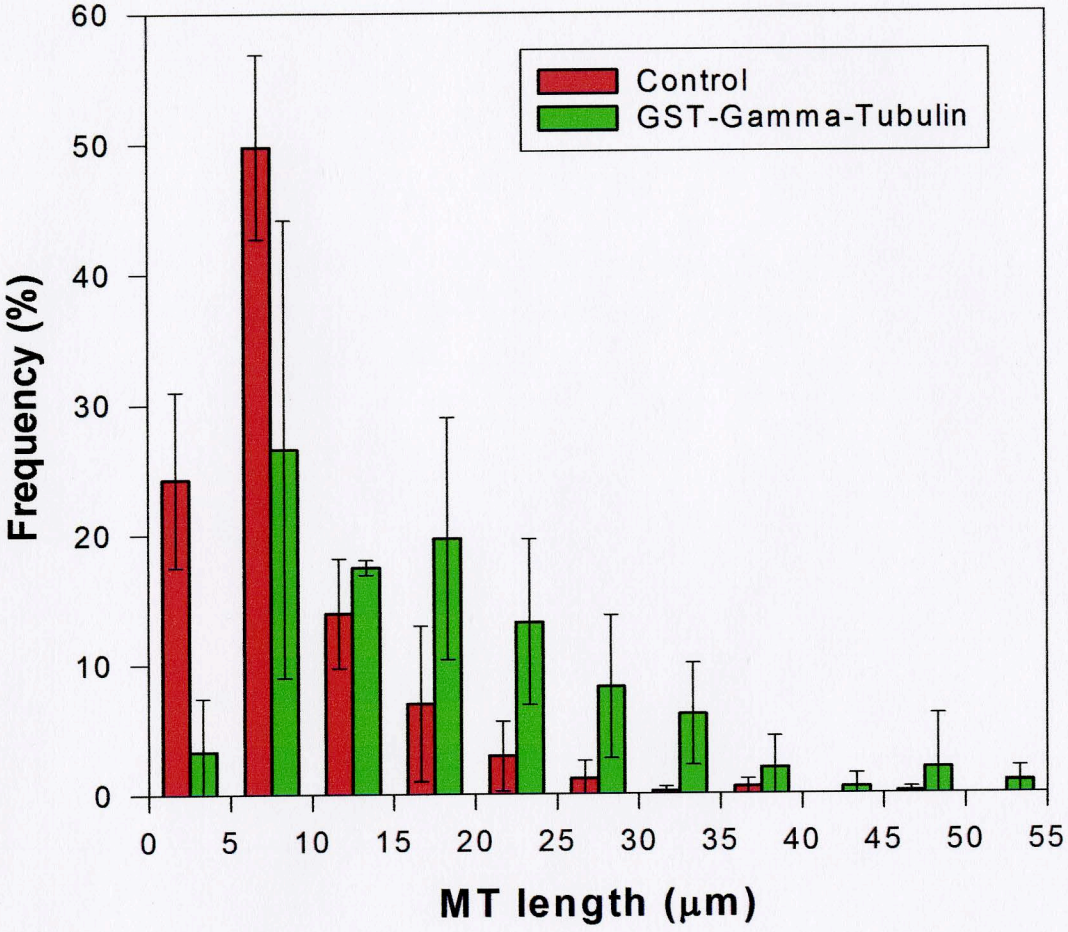
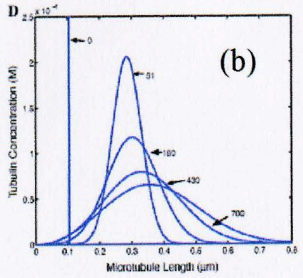


Figure 2 – (a) The graph shown above represents a length distribution for 0.08mg/mL purified tubulin polymerized for 30 minutes at 37°C in PEM buffer (80mM Na-PIPES pH6.9, 1mM MgCl2, 1mM EGTA), 0.21mM GTP, and 16.7uM Taxol alone (A) and with 0.008mg/mL GST-γ-Tubulin (B). The error bars represent the standard deviation of the average microtubule length between trails for each treatment group. The microtubules grew longer in the GST-gamma-tubulin treated groups. This supports a shortening of the lag phase caused by the nucleating effect of GST-gamma-tubulin. The microtubules also reached longer lengths suggesting either a faster growth rate or a stabilizing effect. This would be consistent with the reported nucleating and elongating effects of unmodified gamma-tubulin on polymerizing microtubules in solution. A similar shift in the length distribution was also observed in computer simulations modeling the effects of seeding. (b) A size distribution of a simulated "seeding" experiment that shows the effect of adding a 1, 5, 10, or 20% weight concentration of microtubule seed to a slowly nucleating reversible microtubule system at a single fixed concentration of tubulin is shown to the right (ref. 17). Note the average length of ~0.3μm increasing to about 0.4μm as more seed is added to the reaction solution.



(*Figure from reference 17)

(b)

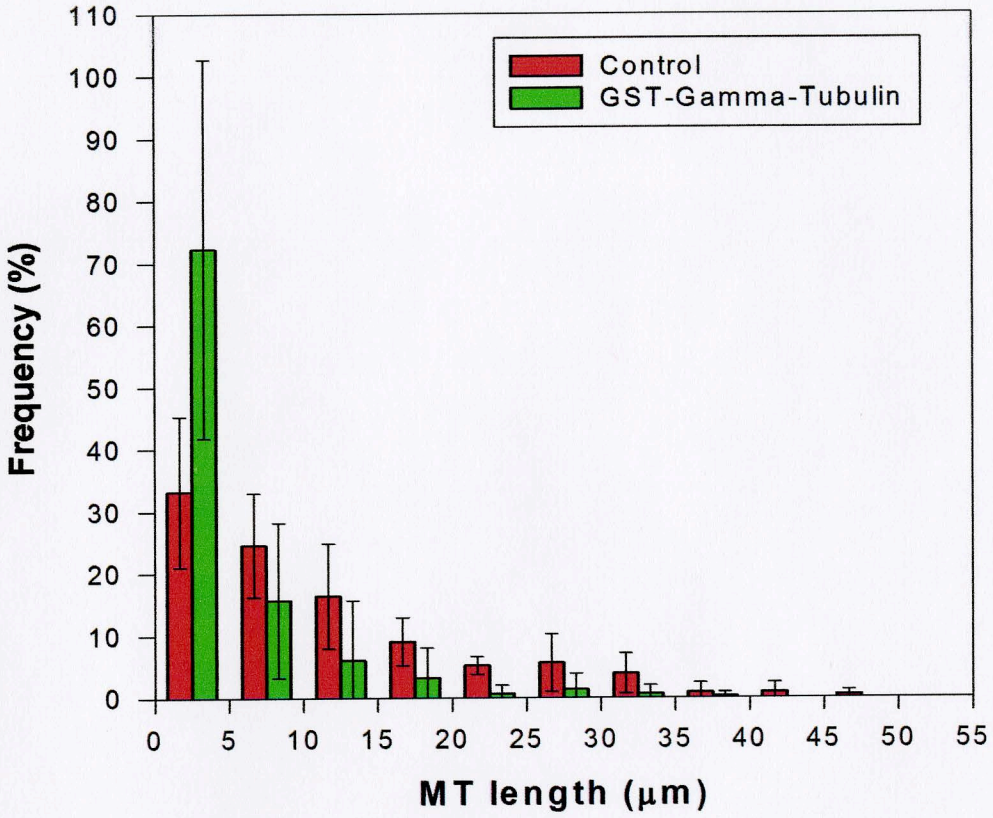
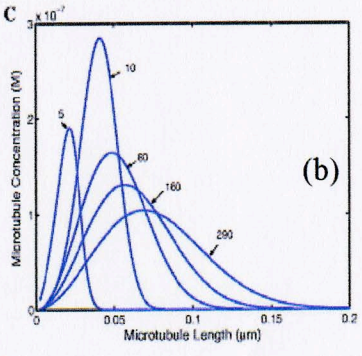


Figure 3 – (a)The above graph represents a length distribution for 1.67mg/mL Purified tubulin polymerized for 30 minutes at 37°C in PEM buffer (80mM Na-PIPES pH6.9, 1mM MgCl2, 1mM EGTA), 0.21mM GTP, and 16.7uM Taxol alone (A) and with 0.008mg/mL GST-γ-Tubulin (B). The error bars represent the standard deviation of the average microtubule length between trails for each treatment group. When microtubules polymerized in higher concentrations of tubulin with GST-gamma-tubulin a higher percentage of them were were short (between 0-5µm) relative to the control groups. We suspect that this is the result of homogenous and heterogenous nucleation proceeding together in parallel to yield a large number of nuclei. The result of distributing the same amount of tubulin dimers amongst a larger number of microtubules is a shorter average length. This is consistent with the effects of unmodified wild type gamma-tubulin on polymerizing microtubules in solution. A similar shift in the length distribution was also observed in computer simulations modeling the effects of a fast nucleating growth environment. (b)The figure to the right shows a size distribution development of a rapidly nucleating reversible microtubule forming system (ref. 17). Note the average microtubule length is only about 0.1µm in the fast nucleation scenario.



(*Figure from reference 17)

length distribution of the microtubules is skewed towards longer lengths (17) (fig 2). With enough nucleating seeds available the free GTP-Tu that is present in solution can be utilized to lengthen the preformed templates. This effect was reproduced with our recombinant GST-γ-tubulin (Figs. 1 and 2) indicating that the recombinant gamma tubulin retains its nucleating capacity.

Figures 1 and 2 represent relatively low concentrations of free tubulin dimer (3.4nM and 1.6nM respectively) compared to the nucleating agent (GST-γ-tubulin, 0.16nM). In this type of situation where there is a surplus of nucleating agent, new microtubules will arise exclusively from heterogenous nucelation. Compared to the control, which is exclusively homogenous nucleation, the GST-γ-tubulin treated groups yielded longer microtubules. Figure 3 represents the length distribution of microtubules polymerized under similar conditions but from a higher initial concentration of tubulin dimer (34nM). When we polymerized microtubules in the presence of GST-γ-tubulin at this higher tubulin concentration we observed shorter microtubules than in the control group. One possible explanation for this is that when enough tubulin is available, heterogenous and homogenous nucleation may occur simultaneously yielding a large number of shorter microtubules relative to a homogenously nucleating control group. This is consistent with predictions made by simulations of microtubule growth modeling fast nucleation conditions (17). It has also been shown that γ-tubulin stabilizes tubulin oligomers by inhibiting GTP hydrolysis (13). More stable oligomers will result in less dissociation and subsequently lower free tubulin concentrations during polymerization. Also, γ-tubulin has been shown to lower the critical concentration for microtubule nucleation. This would prolong the homogenous nucleation

phase of microtubules in the GST-γ-tubulin group, ultimately resulting in a greater number of shorter microtubules.

Plus End Capture *In Situ* - V

A. Plus End Tracking MAPs

The number of known MAPs has been increasing over the years as new proteins are identified. +TIPS are a subfamily of MAPs that are found localized to the plus end of growing microtubules. Within this group are proteins like APC and Tea1p that are translocated to the plus end by a kinesis-based motility, and "plus-end-tracking" proteins that are thought to concentrate at the plus end of growing microtubules by copolymerizing with tubulin heterodimers (18). From the latter group we selected three proteins as plus-end anchoring candidates.

Recombinant fragments of CLIP-170, EB1, and $p150^{glued}$ were engineered previously and are evaluated here as potential components of the plus-end surface adhesion complex. Plus-end-tracking proteins do not preferentially bind microtubule ends *in vitro* (19). Instead they form co-complexes that copolymerize with tubulin heterodimers onto growing microtubule plus ends.

B. Structure and Function of CLIP-170

CLIP-170 is a 55kDa microtubule-organelle linker protein with two functional domains separated by a 950 amino acid stretch of heptad repeats (20). This region is α-helical in nature, and forms a 2-stranded coiled coil that mediates dimerization of CLIP-170 in solution (20). The N-terminal domain binds to microtubules *in vitro* with a stoichiometry of one head domain to two tubulin heterodimers, or one dimer head domain to four tubulin heterodimers (19,20). This domain also has been found to be essential for binding microtubules *in vivo* (21). The C-terminal domain contains two predicted metal binding motifs and is involved in binding microtubules to peripheral cytoplasmic structures (21). Unbound CLIP-170 is auto-inhibited by the

binding of its C-terminal domain to its N-terminal domain (19,22). $p150^{glued}$ and EB1 can relieve this inhibition by binding to the C-terminal and N-terminal domains of CLIP-170 respectively (19,22). Activated CLIP-170 localizes to the plus end of growing microtubules (23) by preassociation with free GTP-Tubulin dimers and copolymerization, followed by rapid regulated release (24,25).

A pool of CLIP-170 is localized to the outer most region of unbound kinetochores, and to the plus end of elongating microtubules during mitosis, where it facilitates the formation of kinetochore-microtubule attachements (26). CLIP-170 binds directly to the $p150^{glued}$ subunit of the dynactin complex via the second metal binding motif of its C-terminal domain (27). This occurs in solution independently of the N-terminal microtubule binding domain (27). CLIP-170 also binds EB1 via its N-terminal domain, forming a co-complex with tubulin heterodimer that copolymerizes into growing microtubules (19).

EB1 was found to be a requisite for plus end localization of CLIP-170 in mammalian cells (28). This could be due to the rapid dissociation of CLIP-170 from plus ends following copolymerization in the absence of EB1. In mutants lacking EB1, CLIP-170 dissociation from the plus ends of microtubules is accelerated (29). In mutants where EB1 is overexpressed and distributed along the length of the microtubule, CLIP-170 continues to localize to the plus end but dissociates more slowly (29).

For our purposes, two recombinant versions of the H2 fragment of CLIP-170 were generated for use as linkers between the surface adhesion complex and the growing plus ends of microtubules in our system. These constructs include both N-terminal CAP-Gly binding domains and part of the heptad repeat chain, but do not include either of the C-terminal metal binding domains. The absence of the C-terminal binding domain in

our recombinant fragments precludes any direct binding of either of our CLIP-170-H2 constructs to either of the recombinant $p150^{glued}$ constructs that were evaluated.

C. Effects of CLIP-170-H2 Constructs *In Situ*

CLIP-170 is known to act as an anti-catastrophe factor to enhance polymerization of microtubules *in vitro* (18, 19, 26, 27). This work evaluates two evaluates two recombinant CLIP-170 fragments, GST-CLIP-170-H2 and HIS-CLIP-170-H2. They are complexed with glutathione-S-transferase(GST) and a short chain of amino acids, respectively.

Overexpression of the microtubule binding domain of CLIP-170 *in vivo* results in bundling of microtubules (25, 27). Figures 4 and 5 illustrate the bundling effect of the HIS-CLIP-170-H2 construct in solution. The H2 constructs appear to group the microtubules together and in some cases form aggregates. The large networks of bundled microtubules appear to be branched. This makes sense considering the role of CLIP-170 as a plus-end tracking protein. The formation of branches is consistent with bundling that is occurring at the polymerizing end of a microtubule.

Figures 6 and 7 show the bundling effects of the GST-CLIP-170-H2 construct. CLIP-170-H2 was shown to increase the proportion of outwardly curled protofilaments at the microtubule plus end (25). It is possible that these protofilaments play a role in the microtubule bundling effect that is observed in the presence of CLIP-170-H2.

The H2 fragment of CLIP-170 induces the formation of tubulin rings in solution (25). Figure 4 demonstrates ring formation induced by the presence of the HIS-CLIP-170-H2 construct. These rings are absent in the GST conjugated construct, presumably due to the steric hinderance caused by the presence of GST. Glutathione-S-transferase is a 30kDa protein occupying significant space relative to the short chain

of histidine residues present on the HIS-H2 construct. Similar findings were reported in other studies of recombinant H2 constructs (25).

The localization of CLIP-170 to Taxol stabilized microtubules is significantly reduced or abolished (27). The microtubules in figure 7 were polymerized with higher concentrations of taxol and lower concentrations of tubulin than the microtubules in figure 6. This explains why the bundling appears to be more tightly clustered in figure 6 and the microtubules in figure 7 appear more elongated with less segmentation.

The morphology of the microtubules observed in these solutions is consistent with what we would expect from solutions containing high concentrations of H2. Taken together, these results confirm that a functional microtubule binding domain has been retained in these recombinant CLIP-170-H2 constructs.

Figure 8 shows the length distribution of microtubules polymerized in the presence of GST-CLIP-170-H2. The microtubules polymerized in the solution containing the H2 fragment grew faster and reached longer lengths. This supports the conclusion that the recombinant H2 constructs have retained the ability to enhance polymerization.

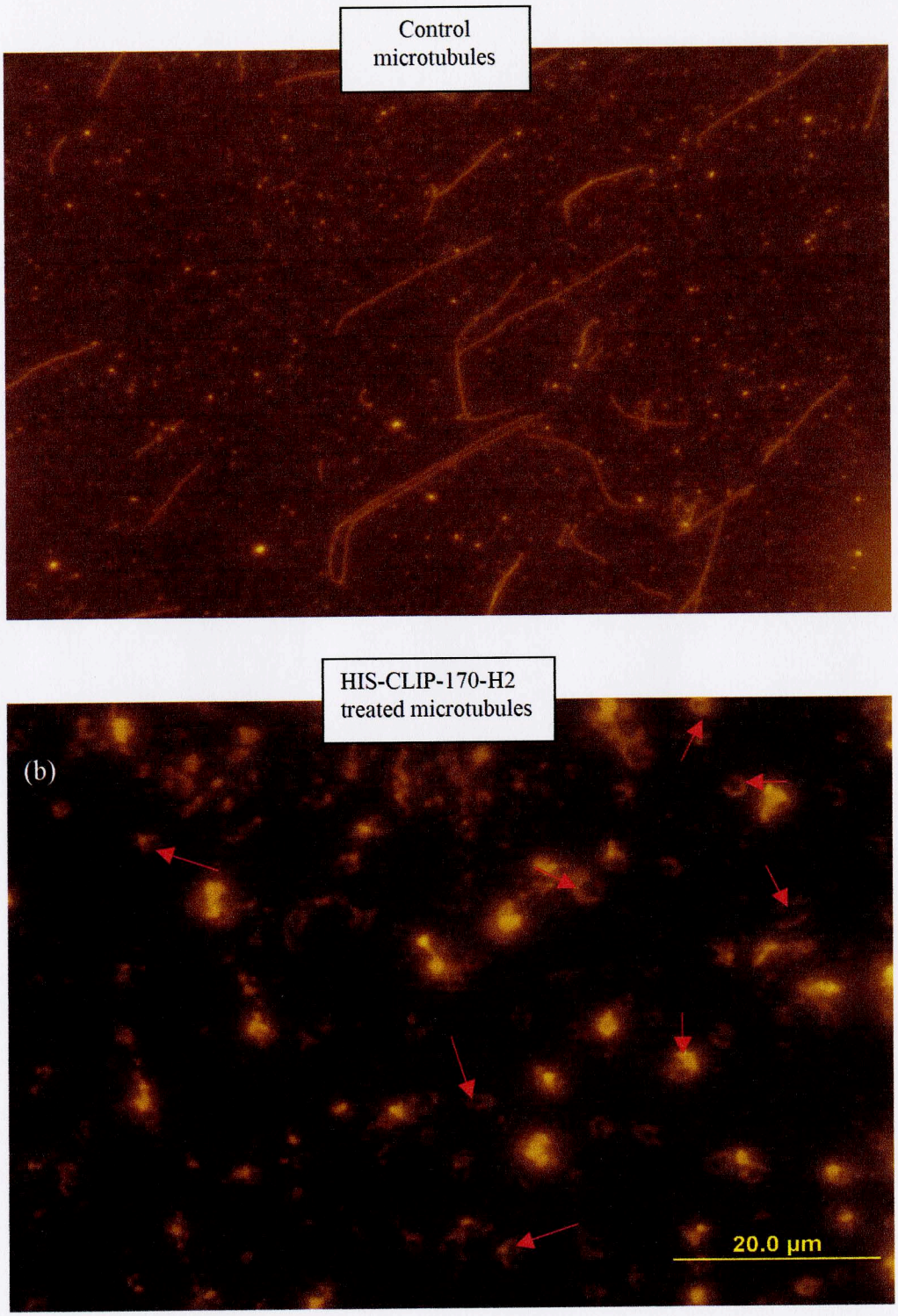


Figure 4 – (a) Control microtubules polymerized from 1.67mg/mL purified tubulin polymerized for 30 minutes at 37°C in PEM buffer (80mM Na-PIPES pH6.9, 1mM MgCl2, 1mM EGTA), 0.21mM GTP, and 16.7uM Taxol are shown on the top. 0.008mg/mL HIS-CLIP-170-H2 is added to the reaction mixture prior to polymerization in the bottom figure (b). The red arrows indicate examples of ring formation that was induced by the HIS-CLIP-170-H2 construct. These rings were not observed in solutions of microtubules polymerized with the GST-CLIP-170-H2 although extensive bundling was seen. We suspect this is due to steric interference from the relatively large 50kDa GST tag.

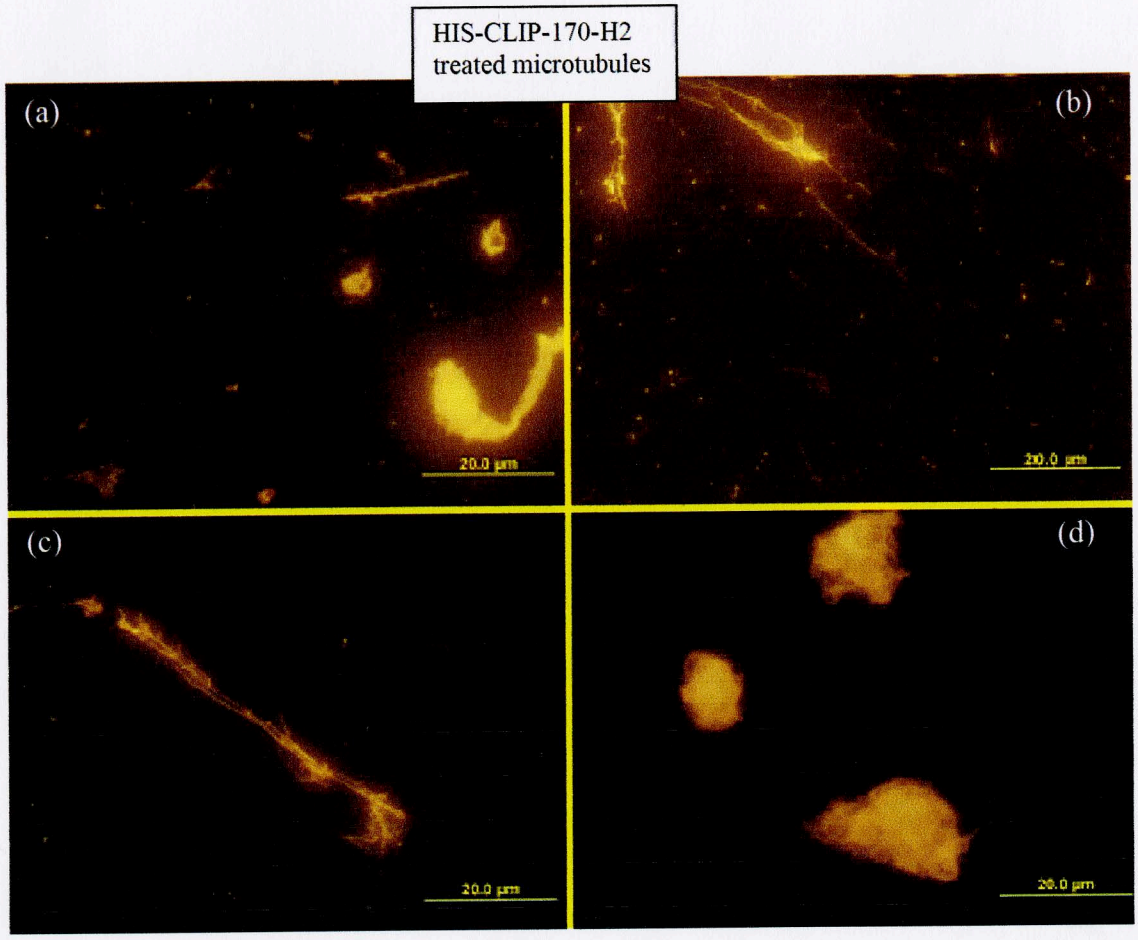


Figure 5 – The images above illustrate the bundling of microtubules polymerized in solutions containing 1.67mg/mL purified tubulin polymerized for 30 minutes at 37°C in PEM buffer (80mM Na-PIPES pH6.9, 1mM MgCl2, 1mM EGTA), 0.21mM GTP, and 16.7uM Taxol with 0.008mg/mL HIS-CLIP-170-H2. The HIS-CLIP-170-H2 fragment induced the formation of (a,d) microtubule clusters and (b,c) microtubule bundles.

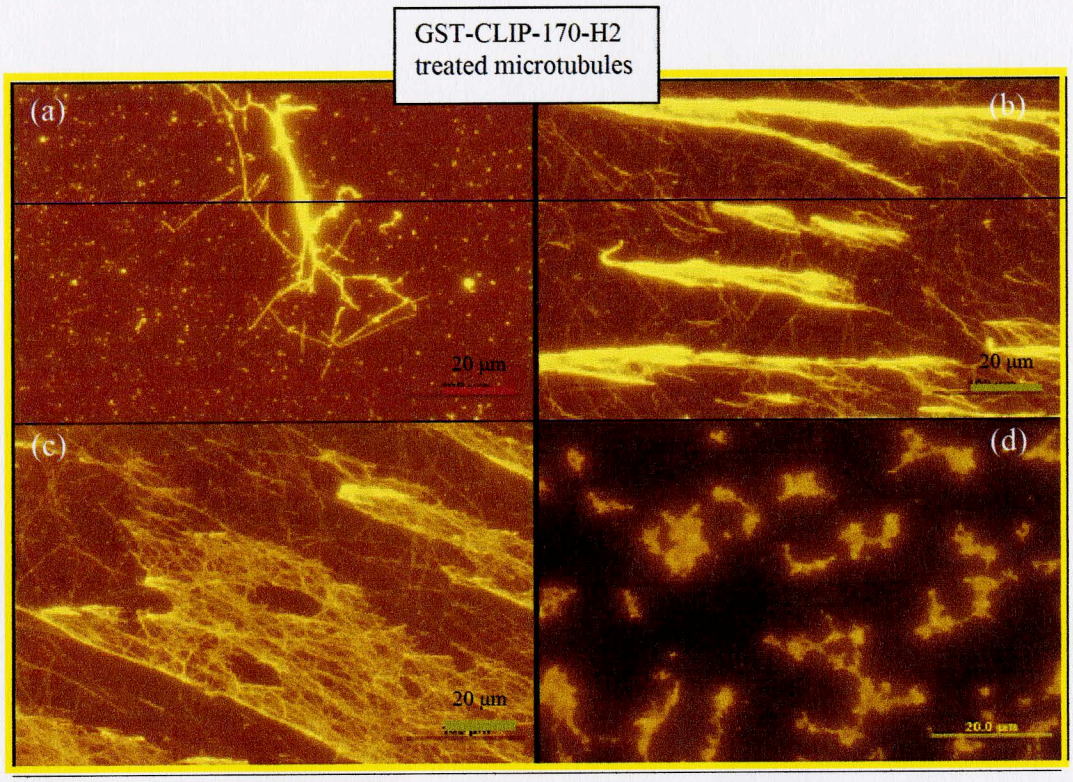


Figure 6 – These pictures show branching and bundling of microtubules polymerized from solutions containing 1.67mg/mL Purified tubulin polymerized for 30 minutes at 37°C in PEM buffer (80mM Na-PIPES pH6.9, 1mM MgCl2, 1mM EGTA), 0.21mM GTP, and 16.7uM Taxol with 0.008mg/mL GST-CLIP-170-H2. Notice the GST-CLIP-170-H2 fragment mainly induced bundling (a), branching (b,c), and some dense clustering (d). Overall there was less aggregate formation relative to the HIS-H2 construct.

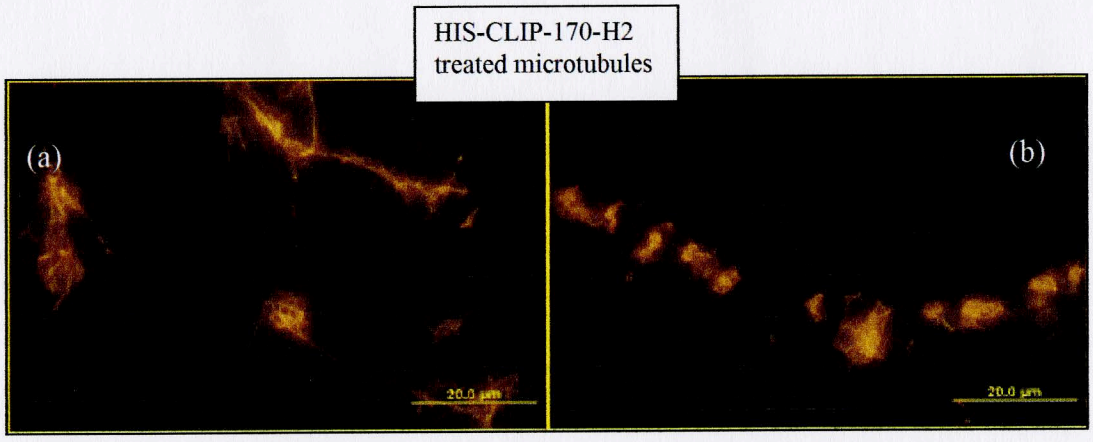


Figure 7 – These images are aggregates of microtubules from solutions containing 0.65mg/mL Purified tubulin polymerized for 30 minutes at 37°C in PEM buffer (80mM Na-PIPES pH6.9, 1mM MgCl2, 1mM EGTA), 0.21mM GTP, and 33.4uM Taxol with 0.008mg/mL HIS-CLIP-170-H2. These solutions contained a lower initial concentration of tubulin dimer and higher concentrations of Taxol than was used in figures 6 and 7. The aggregates (a,b) appear to be less dense with less branching than those formed with the GST-H2 construct. This suggests that the presence of Taxol is interfering with the plus end activity of the H2 fragments.

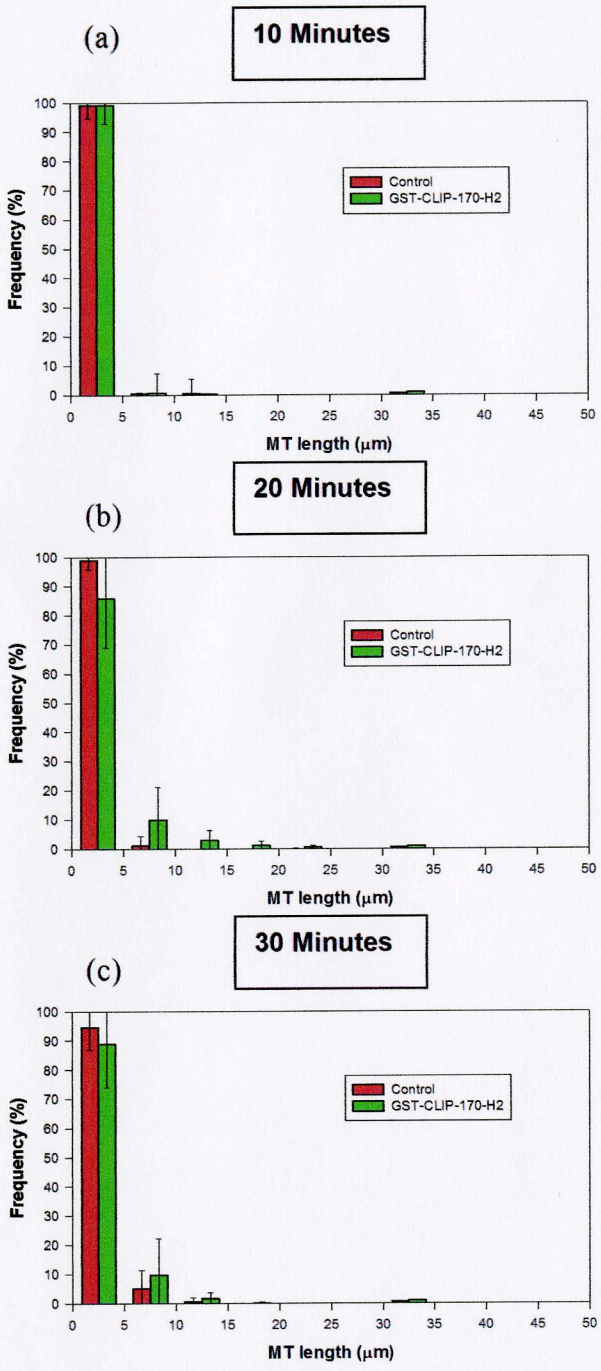


Figure 8- The above graphs show length distribution for microtubules polymerized in solutions containing 0.17mg/mL purified tubulin polymerized at 37°C for 10 (a), 20 (b), and 30 (c) minutes in PEM buffer (80mM Na-PIPES pH6.9, 1mM MgCl2, 1mM EGTA), 0.21mM GTP, and 16.7uM Taxol alone (A) and with 0.008mg/mL GST-CLIP-170-H2. The error bars represent standard deviation of the average microtubule length between trails for each treatment group. The presence of microtubules >5μm after 10 minutes of polymerization in the GST-CLIP-170-H2 treated groups indicates a slight nucleating effect. After 30 minutes the GST-CLIP-170-H2 groups contained longer microtubules. This confirms published findings that CLIP-170-H2 acts to enhance polymerization.

D. Structure and Function of EB1 and p150^{glued}

EB1 is a 30kDa microtubule plus-end-tracking protein that binds CLIP-170 (19), centrosomes, the tumor supressor protein adenomatous polyposis coli (APC), and p150^{glued} (30). It contains an N-terminal CH domain that is involved in binding microtubules, and a C-terminal motif associated with APC and p150^{glued} binding (30) and centrosomal localization (31). p150^{glued} is a 150kDa dimeric protein that is involved in centrosomal anchoring (31) and transport (32). It binds dynein to form a minus end directed microtubule motor called the dynactin complex.

EB1 exhibits an auto-inhibiting structure in its unbound state similar to CLIP-170. The C-terminal domain binds to the N-terminal domain, sterically blocking access to the microtubule binding site of EB1. Binding of the C-terminal domain to p150^{glued} releases the N-terminal domain allowing EB1 to interact with microtubules (22). CLIP-170 and p150^{glued} compete with each other for binding to the C-terminal domain of EB1 (19).

This competitive inhibition makes sense considering the differential role of these proteins during mitosis. An interaction between p150^{glued} and EB1 is required for minus end anchoring of microtubules to the centrosome and is necessary for the elongation of a radial microtubule array from the centrosome to proceed (31). Elongating spindle microtubules incorporate CLIP-170 into their plus ends through a copolymerization mechanism. EB1 binding to p150^{glued} may act as both a checkpoint for minus end anchoring of microtubules and a signal initiating astral microtuble elongation by relieving the auto-inhibitory function of EB1. Since the EB1/ p150^{glued} interaction is independent of the microtubule binding domain, it is possible that cytoplasmic association of these two proteins facilitates their

colocalization to the minus end of nucleating microtubules. Once anchoring has taken place and EB1's minus end role has been fulfilled, CLIP-170 levels could replace $p150^{glued}$ at the C-terminal through competitive binding and facilitate its transport to the plus end where it promotes elongation. Relief of CLIP-170 auto-inhibition may promote this transition by sequestering $p150^{glued}$ following its release from EB1. Recall that CLIP-170 and $p150^{glued}$ both naturally exist as dimers in solution, allowing them to complex with each other and EB1. As the microtubule lengthens, the co-complexes of these three proteins localize at the growing plus end where they facilitate kinetochore-microtubule attachment and positioning.

E. Effects of EB1 and $p150^{glued}$ Constructs *In Situ*

EB1 has been shown to exhibit promotion of microtubule rescue and inhibition of catastrophe resulting in enhanced elongation of microtubules (18, 19, 32, 33). Subsequent analysis of these findings suggests that the microtubule elongating effects of EB1 may depend on the binding of APC or another activating protein (31, 32, 34). $p150^{glued}$ has been shown to nucleate microtubules *in vitro* by lowering the critical concentration (33). A separate study reported conflicting results, claiming that $p150^{glued}$ did not nucleate microtubule polymerization in the absence of seeds (22). The role of $p150^{glued}$ as a regulator of microtubule polymerization *in vitro* remains unclear at the present.

To evaluate this effect, length distributions for microtubules polymerized *in vitro* were obtained for low and high concentrations of recombinant EB1 and $p150^{glued}$ constructs (Figs 9 and 10 respectively). The recombinant proteins did not demonstrate a significant effect on

Low Concentration End-Capping Proteins

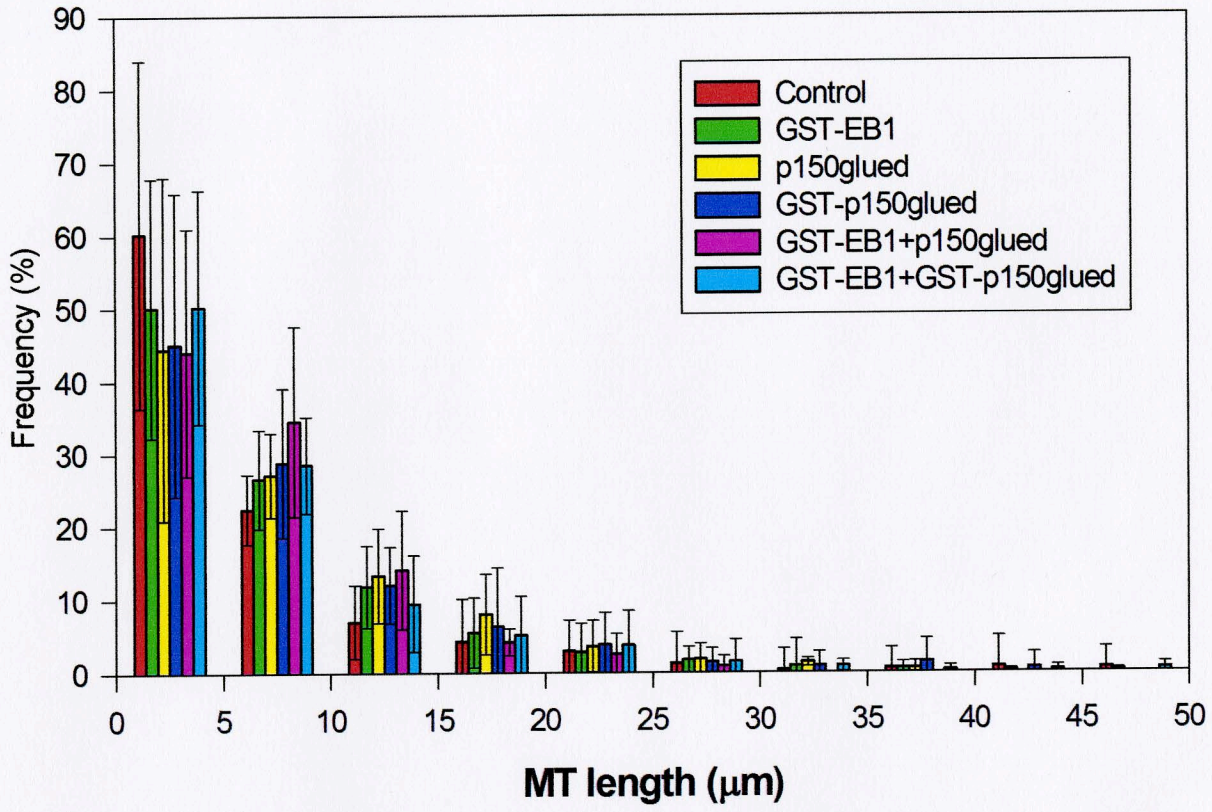


Figure 9 – This graphs shows length distributions for 0.33mg/mL(7μM) Purified tubulin polymerized for 30 minutes at 37°C in PEM buffer (80mM Na-PIPES pH6.9, 1mM MgCl2, 1mM EGTA), 0.21mM GTP, and 16.7uM Taxol alone (**A**) and in combinations with 0.09mM GST-EB1 (**B**), 0.13mM $p150^{glued}$ (**C**), 0.33mM GST- $p150^{glued}$ (**D**), 0.09mM GST-EB1 + 0.13mM $p150^{glued}$ (**E**), and 0.09mM GST-EB1 + 0.33mM GST-$p150^{glued}$ (**F**). The error bars represent the standard deviation of the average microtubule length between trails for each treatment group. At this concentration there is no statistically significant effect on polymerization by the plus-end constructs.

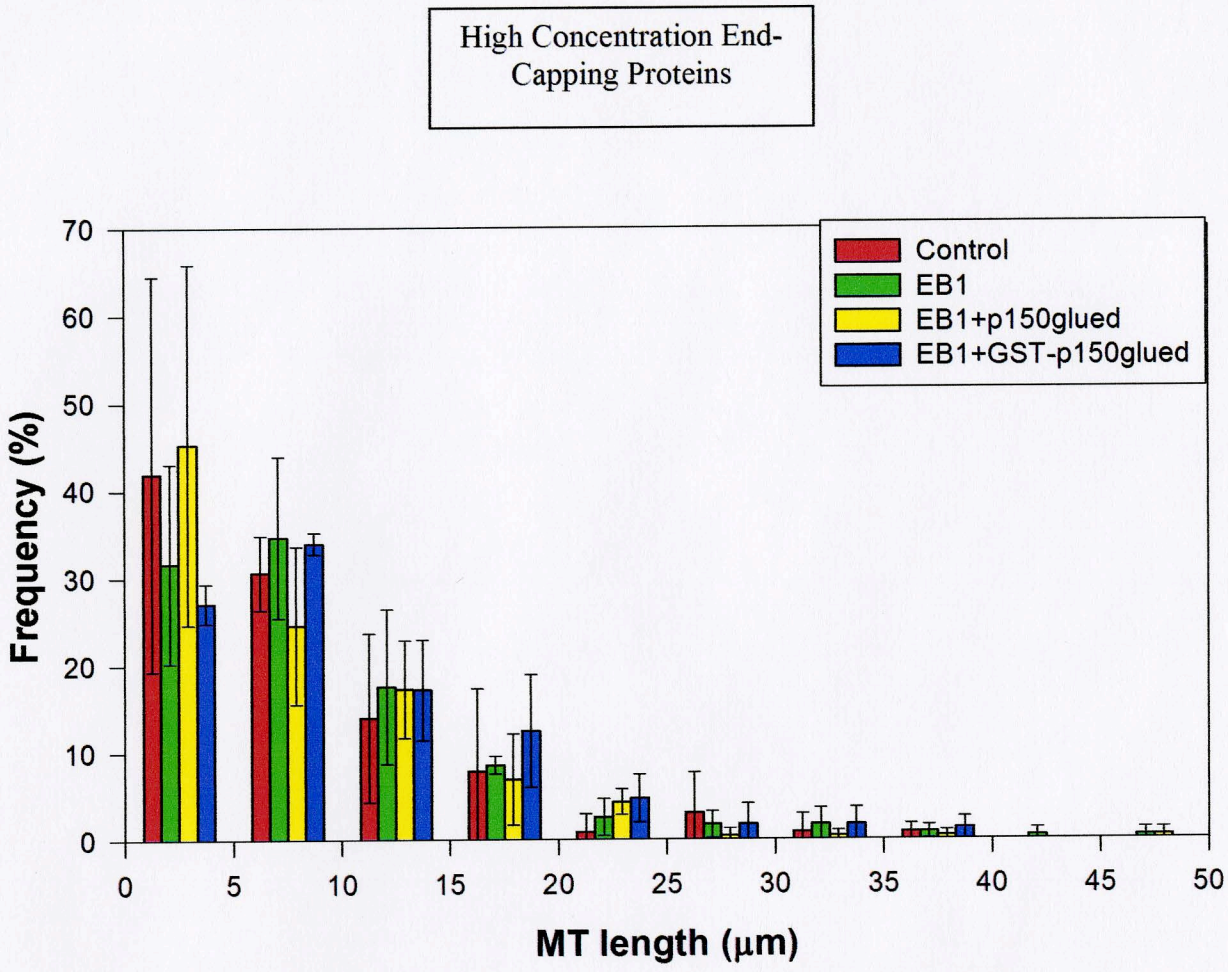


Figure 10 - Length distribution for 0.33mg/mL(7μM) Purified tubulin polymerized for 30 minutes at 37°C in PEM buffer (80mM Na-PIPES pH6.9, 1mM MgCl2, 1mM EGTA), 0.21mM GTP, and 16.7uM Taxol alone (**A**) and in combinations with 4.6mM EB1 (**B**), 1.3mM $p150^{glued}$ + 4.6mM EB1 (**C**), and 3.3mM GST- $p150^{glued}$ + 4.6mM EB1 (**D**). At higher concentrations relative to figure 9 there is still no statistically significant effect on polymerization by the plus-end constructs. The presence of Taxol in the reaction solution may inhibit plus-end tracking MAP activity, masking any growth effects caused by the capping protein constructs.

the length distributions of microtubules polymerized in their presence. The localization of both EB1 and p150^{glued} to Taxol stabilized microtubules was significantly reduced or abolished (27). The use of Taxol in generating these length distributions may explain why no significant elongation was observed in the treatment groups that combined the EB1 and p150^{glued} constructs. This may also explain the lack of apparent nucleation by the treatment groups containing p150^{glued} constructs alone.

EB1 and p150^{glued} are known to induce microtubule bundling upon overexpression (33, 31). The bundled microtubules resulting from increased levels of EB1 are longer than control, while microtubules polymerized with increased levels of p150^{glued} are shorter than control (33). The resulting bundles were shown to be very stable and resistant to the microtubule destabilizing agent nocodazol.

Solutions of preformed rhodamine-tubulin microtubules were diluted in PEM buffer containing the EB1 and GST-p150^{glued} constructs and observed over the course of 10-15 minutes (figs 11 and 12). After 2-3 minutes all of the microtubules in the solution had dissociated beyond a detectable limit. The EB1/p150^{glued} constructs did not inhibit disassembly following the dilution as we had predicted. It is possible that the disassembly brought on by the dilution exceeded the capacity of the EB1/p150^{glued} complex to inhibit catastrophe. Another possibility is a loss of functionality due to the lingering presence of Taxol in the dilution. The concentration of Taxol in the dilution solution was about 1μM.

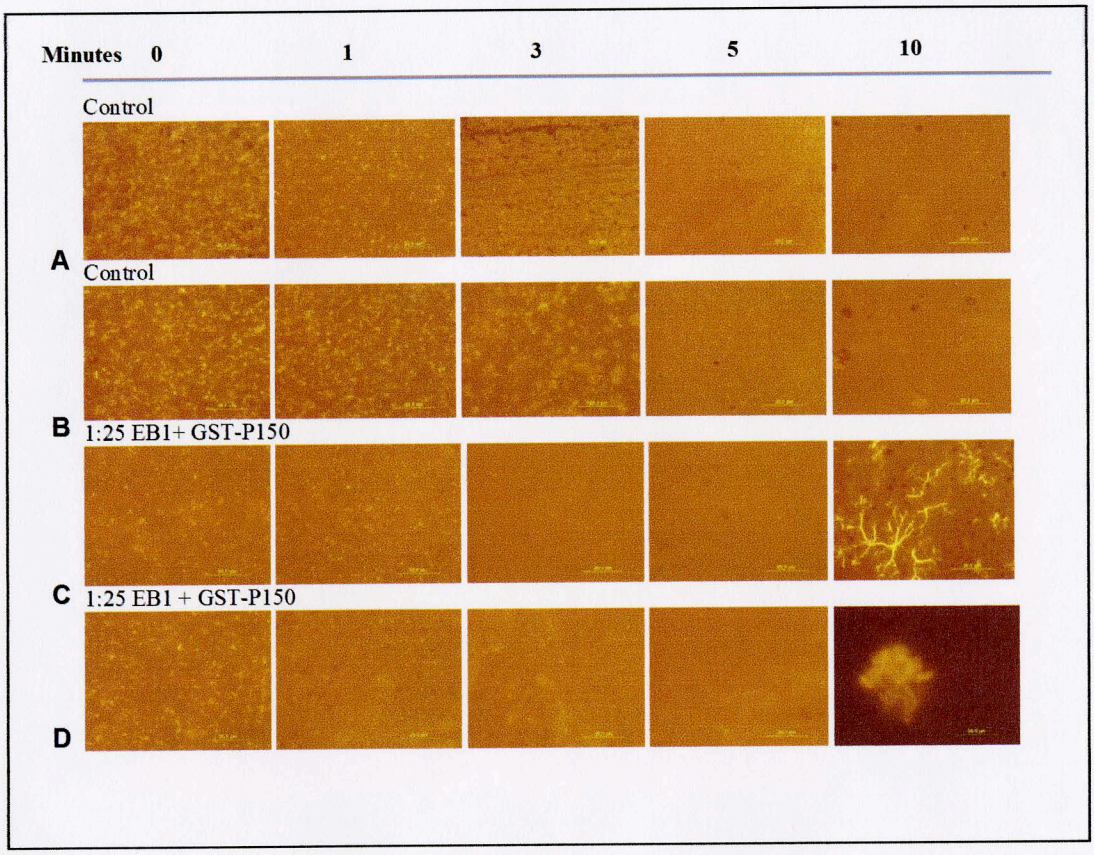


Figure 11 – A microtubule stock solution containing 7 μM tubulin was polymerized for 30 minutes at 37°C in PEM buffer (80mM Na PIPES pH6.9, 1mM MgCl2, 1mM EGTA). 0.23mM GTP, and 46μM Taxol. The microtubule stock solution was then diluted 1:50 in PEM (A and B) and PEM containing 1.84mM EB1+1.32mM GST-p150^{glued} (C and D). Images were obtained 1,3,5, and 10 minutes following the dilution. The microtubules were mostly all gone after three minutes in the diluted solution. At ten minutes aggregates appeared in the solutions containing EB1 and p150 but not in the control groups, suggesting that the recombinant protein fragments induce bundling and/or growth in solutions that are too dilute to otherwise support polymerization.

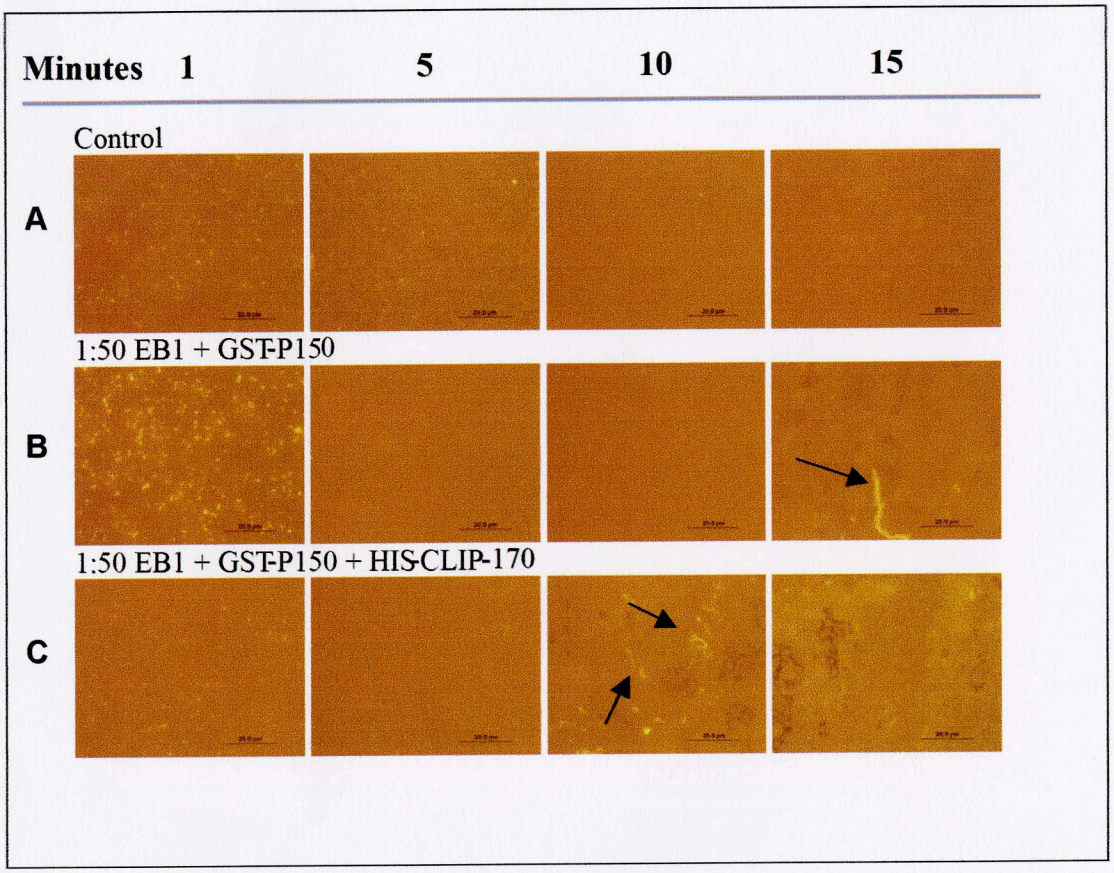


Figure 12 – 7 μM tubulin was polymerized for 30 minutes at 37°C in PEM buffer (80mM Na PIPES pH6.9, 1mM MgCl2, 1mM EGTA). 0.23mM GTP, and 46μM Taxol to yield a microtubule stock solution. The stock solution was diluted 1:50 in PEM (A) and PEM containing 0.92mM EB1+0.67mM GST-$p150^{glued}$ (B) and PEM containing 0.92mM EB1+0.67mM GST $p150^{glued}$+38μM HIS-CLIP-170-H2 (C). Images were then obtained at 1,5,10, and 15 minutes after the dilution. At five minutes the microtubules had completely disappeared. Arrows indicate aggregates that appeared in the groups containing EB1 and p150 but not in the control. The aggregates formed sooner when the CLIP-170-H2 fragment was in the dilution solution, suggesting that H2 may enhance the effect of EB1 and p150.

After a lag period of about 10 minutes from the initial dilution large aggregates of tubulin appeared in the groups containing recombinant proteins, but were absent from the control groups. This at least proves that the recombinant proteins have retained some ability to bind and bundle microtubules. It is possible that the rescuing properties of EB1 and nucleating capacity of $p150^{glued}$ also contributed to the formation of the observed aggregates. However, it is equally likely that no nucleation or growth occurred during the lag phase. Instead, disassembly could have shortened the microtubules to a length that was undetectable in the light microscope pending sufficient bundling into larger complexes. Interestingly, when HIS-CLIP-170-H2 was added to the dilution buffer containing EB1 and $p150^{glued}$ a reduction in the lag period preceding the appearance of the aggregates was observed (fig 12).

Taken together, these results demonstrate that the recombinant plus-end-tracking protein constructs interact with microtubules *in situ,* significantly altering tubulin polymerization dynamics and microtubule morphology. The observed effects are consistent with the known properties of their unaltered wild type analogs.

Surface Immobilization - VI

A. SAM Complex

After confirming the capacity of the recombinant CLIP-170-H2 constructs to bind and bundle microtubules, we used the H2 fragment that was conjugated to glutathione-S-transferase (GST) to evaluate the integrity of our surface adhesion complex. Figure 13 shows a sharp contrast in fluorescence between two SAM functionalized wafers following exposure to tubulin heterodimer and subsequent anti-tubulin immunolabeling. The lower wafer was exposed to 0.05mg/mL GST-CLIP-170-H2 and the upper wafer was not. The bright fluorescence emitted by the lower wafer relative to the control wafer above it indicates that the anti-GST surface adhesion complex effectively bound the GST-CLIP-170-H2 construct, which in turn bound the tubulin heterodimer and emitted fluorescence. This supports the retention of a viable microtubule binding domain in the H2 construct, confirms the association of conjugated glutathione-S-transferase to anti-GST antibody, and validates the presence of a working surface adhesion complex. Previous work confirmed these findings by binding a recombinant GST-γ-tubulin construct to fluorescently labeled GFP-anti-γ-tubulin antibody and verified the viability of each element in the surface adhesion complex using fluorescent microscopy (35).

B. Microtubule Anchoring From Functionalized Surfaces

Gold plated silica wafers were functionalized with SAM of surface adhesion complexes (MHA) bound to anti-GST. These surfaces were exposed to either GST-γ-tubulin or GST-p150^{glued} and evaluated for their ability to immobilize microtubules at their ends. Fig. 14 shows control surfaces functionalized with the SAM but without the recombinant capping protein were exposed to polymerizing microtubules

to compare with the GST-gamma-tubulin surfaces and pre-polymerized microtubules to compare with the plus-end-tracking surfaces.

The GST-γ-tubulin functionalized surfaces were exposed to the tubulin solution during polymerization in order to allow for nucleation to take place from the surface bound γ-tubulin constructs.

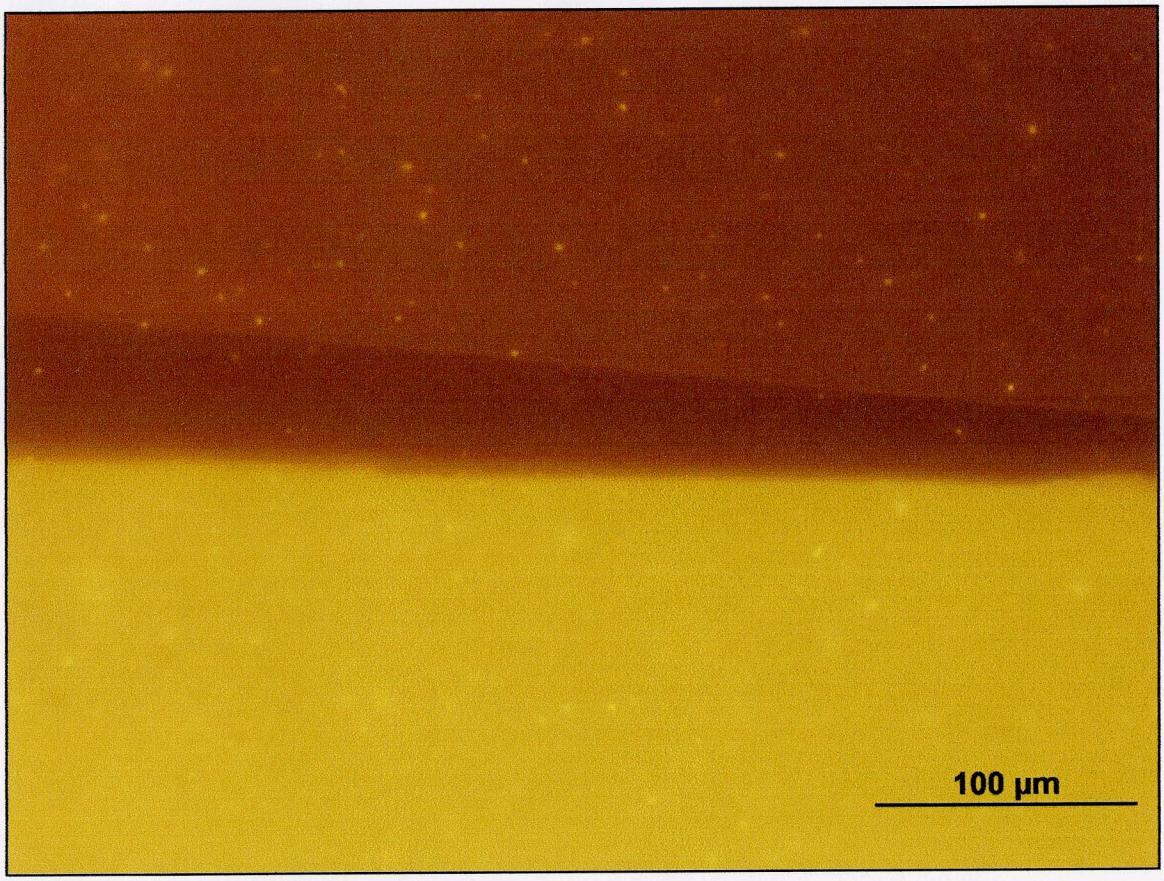


Figure 13 – Two gold plated silica surfaces functionalized with anti-GST bound surface adhesion complexes. The lower surface was exposed to 0.05mg/mL GST-CLIP-170-H2 while the upper surface was not. Both surfaces were then incubated with tubulin heterodimer and immunolabeled with 1° antibody specific to β-tubulin Cy3 2° antibody antibody. The sharp contrast in fluorescence indicates successful binding of tubulin dimer to the functionalized surface.

Figure 15 shows a microtubule that is anchored to the SAM-γ-tubulin functionalized surface by its minus end, while the distal plus end floats freely in solution. In comparison to the controls in figure 14, Figure 15 clearly indicates MT binding to the GST-γ tubulin fusion protein. In figure 16 the microtubules indicated by the arrows are out of the surface plane of focus at one end. This corresponds to what would be expected from a microtubule that was anchored to the surface at one end and protruding outward normal to the surface of the wafer at the other end.

Preformed microtubules were incubated with recombinant EB1 during the last ten minutes of polymerization to ensure localization of the EB1 construct to the dynamic plus end. These were then added to the GST-p150^{glued} functionalized wafers to simulate a plus end capture scenario. Figure 17 shows the anchored plus end of the microtubule in focus at the left, the center region in focus in the middle, and the free minus end in focus well above the surface of the functionalized surface. Figure 18 shows other examples of microtubules that have been captured and immobilized to the surface at their plus end, while the minus end remains unbound and out of the plane of focus. Comparison with fig. 14c, shows clearly activity of the p150-EB1 as a binding complex. Interestingly, Fig. 14d also shows MT binding to the p150 functionalize surface in the absence of EB1 suggesting that EB1 may not be necessary for plus end binding.

The surfaces that were functionalized with GST-γ-tubulin (figs 15,16) appear to have significantly large amounts of background signal. This effect may be the result of small microtubule nuclei beyond the resolution limit of the microscope. As is the case with the formation of microtubule asters, nucleation of many microtubules from a

stationary nucleation center results in a local depletion of free tubulin. The rate of diffusion of tubulin dimer in the solution governs the size of this depletion zone. The microtubules within this zone will undergo dynamic instability while competing with one another for free tubulin dimer. The competition between microtubules limits the amount of dimer

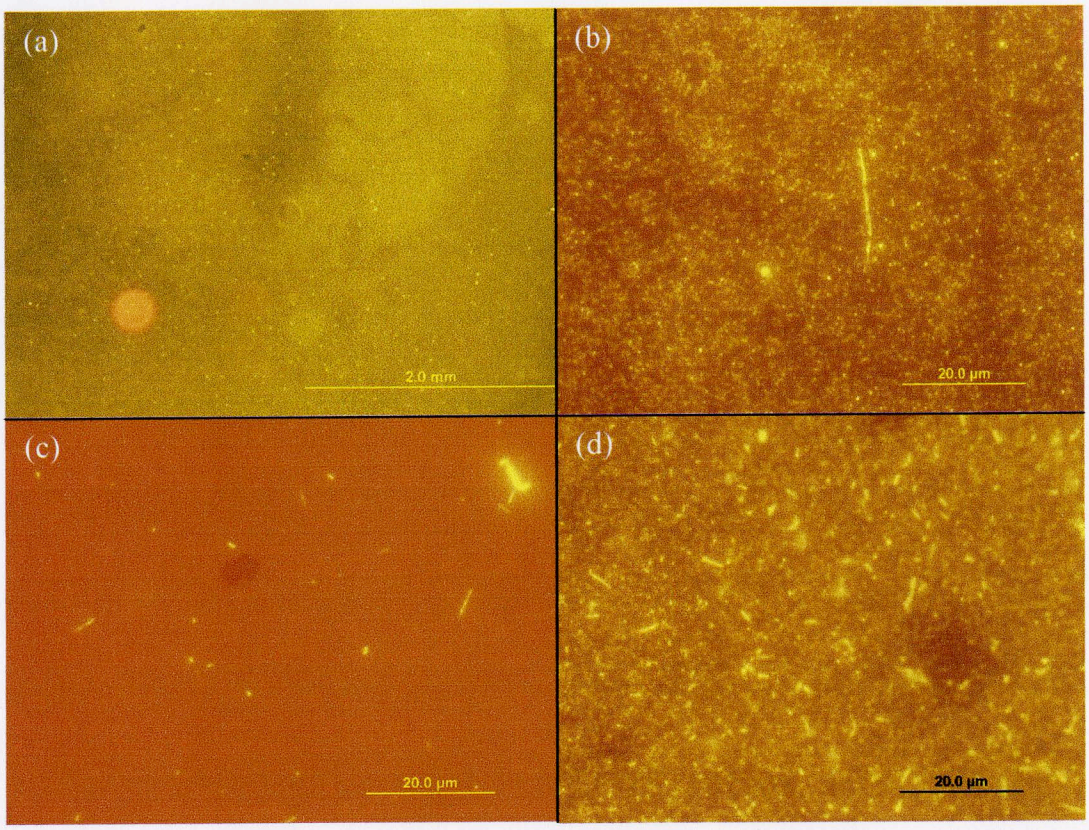


Figure 14 - Images of control surfaces showing (a) an MHA functionalized surface submerged in microtubule stock solution (7µM tubulin + 10mM GTP + 2mM Taxol in PEM buffer) during polymerization, (b) an anti-GST functionalized surface submerged in microtubule stock solution (7µM tubulin + 10mM GTP + 2mM Taxol in PEM buffer) during polymerization, (c) an MHA functionalized surface submerged in pre-polymerized, EB1-treated, microtubule stock solution (7µM tubulin + 10mM GTP + 2mM Taxol in PEM buffer heated to 37°C for 20 minutes before adding 0.008mM EB1 and heating to 37°C for another 10 minutes), and (d) a p150 functionalized surface submerged in pre-polymerized microtubule stock solution (7µM tubulin + 10mM GTP + 2mM Taxol in PEM buffer). Controls (a), (b) and (c) show essentially no anchoring of MTs to the surface. Numerous MTs are anchored by one end to the surface in (d).

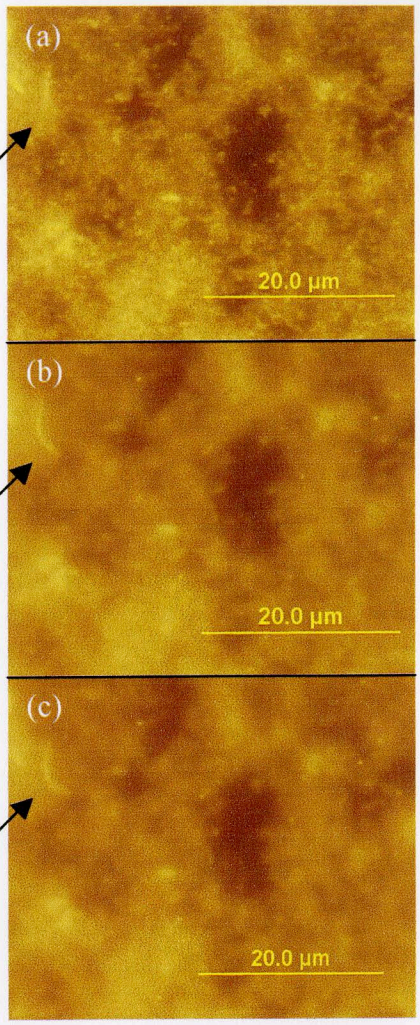


Figure 15 – A minus end microtubule is shown bound to a GST-gamma-tubulin functionalized Au surface. The image on the top (a) shows the anchored end in focus with an arrow pointing to the unbound end. The next two images (b,c) are focused at the unbound end and show it waving side to side in the solution.

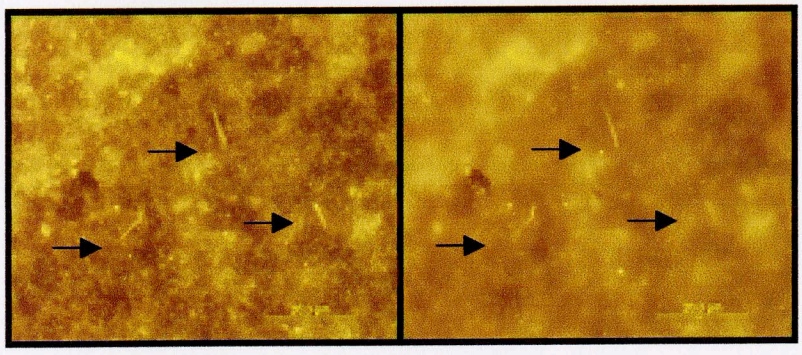


Figure 16 – The above images show three microtubules bound to a GST-gamma-tubulin functionalized gold surface. The anchored end is in the plane of focus in the image on the left (a). The unbound plus end is show in focus in the image on the right (b) while the other end is out of focus. This implies that microtubule is positioned perpendicular to the surface plane.

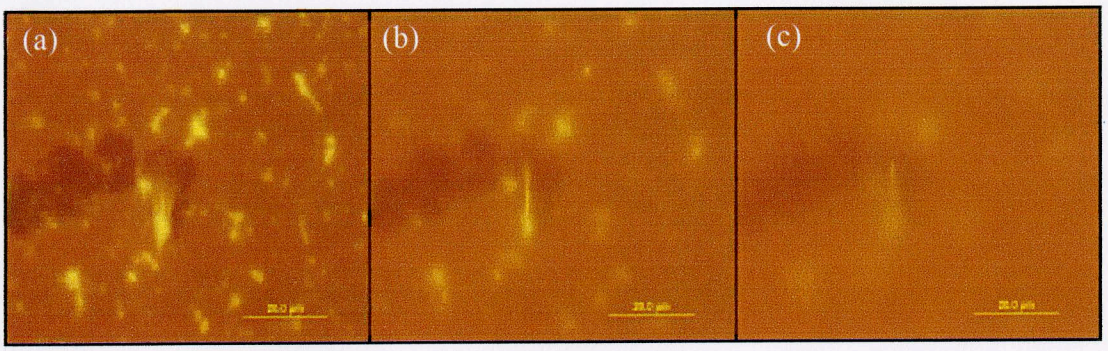


Figure 17 – This series shows three focal planes of an EB1 bound microtubule anchored to a p150 functionalized surface. The anchored plus end is indicated by the arrow while the plus end (a), middle (b), and unbound minus end (c) are in the focal plane. **The above the image series at the top of the page is an image of a blank control surface for comparison.

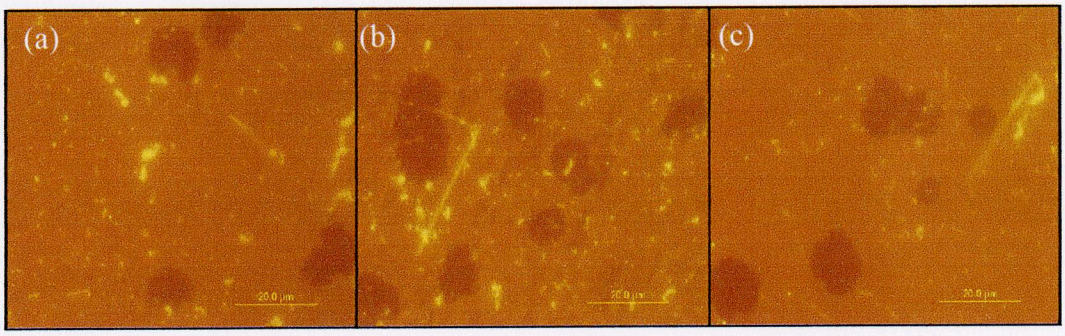


Figure 18 – Arrow in the images above point to three different EB1 bound microtubules captured onto p150 functionalized surfaces. The arrow indicates the anchored end while the opposite end is unbound and out of focus.

available for growth resulting in high rates of catastrophe relative to elongation. Microtubules growing past the depletion zone will escape this catastrophe cycle. The dimer concentration outside the depletion zone is high, so escaping microtubules will generally grow very long relative to solution microtubules polymerized in similar conditions. This is a growth phenomenon and is therefore absent in the $p150^{glued}$ capturing surfaces.

Conclusions - VII

We have analyzed the nucleating capacity of GST-γ-tubulin using length distributions of microtubules polymerized in solution. At low initial tubulin concentrations, GST-γ-tubulin eliminated the lag phase corresponding to homogenous nucleation. It also enhanced the growth phase, yielding longer microtubules. This is consistent with simulations of microtubule polymerization in the presence of a large proportion of nucleating seeds relative to tubulin dimer. At high initial tubulin concentrations, GST-γ-tubulin treated groups yielded many short microtubules. This is consistent with simulations and observations of solutions containing a surplus of tubulin and a fast nucleating agent. Taken together, these findings suggest that the recombinant GST-γ-tubulin construct is functional as a microtubule nucleating agent and as an inorganic surface ligand.

We examined the effects of HIS-CLIP-170-H2 and GST-CLIP-170-H2 on microtubule polymerization *in situ*. Both of the recombinant CLIP-170-H2 constructs that were evaluated demonstrated significant bundling at high concentrations in solution. The histidine tagged construct induced ring formation in solution while the GST bound construct did not. This is likely due to steric hinderance caused by the relatively large size of the GST domain. While both constructs appear to have retained their interaction with microtubules, HIS-CLIP-170-H2 does not appear to be impaired structurally like GST-CLIP-170. In solutions of diluted microtubules, the EB1 and p150 constructs demonstrated aggregate formation following depolymerization. This bundling effect implies an ability to bind microtubules, which was enhanced by the presence of the HIS-CLIP-170-H2 construct. We conclude that all of the recombinant plus end tracking proteins have retained their ability to

bind microtubules and are therefore suitable for use as inorganic surface ligands.

GST-γ-tubulin and GST-p150 with or without EB1 were shown to be able to anchor microtubules to functionalized gold surfaces, supporting our conclusion that these recombinant end-capping proteins are effective inorganic surface ligands.

APPENDIX A - Materials and Methods - VIII

Preparing GTP Aliquots (10mM GTP in Water):

1. Pipet 1mL H_2O into tube of lyophilized GTP powder and vortex to mix.
2. Prepare 10µL aliquots in labeled tubes and store at -20°C.

Preparing Taxol Aliquots (2mM Paclitaxel in DMSO):

1. Resuspend lyophilized powder in 100µL DMSO and vortex to mix.
2. Prepare 10µL aliquots in labeled tubes and store at -20°C.

Preparing Tubulin Aliquots (10mg/mL Tubulin in PEM buffer):

1. Prepare liquid nitrogen bath
2. Resuspend lyophilized powder in 100µL PEM buffer and pipet to mix.
3. Prepare 10µL aliquots in labeled tubes.
4. Snap freeze tubes by dropping them in liquid nitrogen while wearing proper protective gloves.
5. Retrieve aliquots using forceps and store at -80°C.

Preparing Primary Antibody Aliquots (200µg/mL mouse α-β-tubulin in 10% glycerol):

1. Prepare liquid nitrogen bath.
2. Pipet 100µL glycerol into 900µL H_2O to make 10% glycerol.
3. Resuspend lyophilized powder in 500µL 10% glycerol and pipet to mix.
4. Prepare 5µL aliquots in labeled tubes
6. Snap freeze tubes by dropping them in liquid nitrogen while wearing proper protective gloves.
5. Retrieve aliquots using forceps and store at -80°C.

Preparing Secondary Antibody Aliquots (750µg/mL goat anti-mouse Cy3 in 50% glycerol):

1. Pipet 1.1mL H_2O into tube of freeze-dried power.
2. Pipet 1.1mL glycerol into tube and pipet to mix.
3. Prepare 10µL aliquots in labeled tubes and store covered at -20°C.

Determining Protein Concentrations:

1. Turn on Nanodrop ND-1000 spectrophotometer.
2. Select Protein A280 mode.
3. Calibrate and blank spectrophotometer.
4. Absorbance reading yields protein concentration in mg/mL.

Microtubule Stock Solution (1.67mg/mL(*33.4μM*) Tubulin, 17μM Taxol, 0.21mM GTP in PEM buffer):

1. Prepare an ice bucket and supply it with PEM buffer, GTP, Taxol, and tubulin.
2. Place an open microcentrifuge tube that has been properly labeled in the ice bath.
3. Pipet 48.25μL PEM buffer into the microcentrifuge tube.
4. Pipet 1.25μL GTP (10mM stock solution) into the microcentrifuge tube.
5. Pipet 0.5μL Taxol (2mM stock solution) into the microcentrifuge tube by removing the tube from the ice bath and holding it up to a light background during pipeting. This is to ensure the delivery of such a small volume. An oily streak should be ejected from the pipet tip.
6. Cap the tube and vortex to mix.
7. Place the tube back in the ice bath and add 10μL tubulin (10mg/mL).
8. Pipet mix with 200μL pipet.
9. Place the tube in a 37°C incubator for 30 minutes.

**For a different concentration of tubulin or additional reagents change the amount of PEM buffer to maintain a final volume of 60μL*

Blocking Solution (2%BSA in PEM buffer):

1. Weigh 1g BSA into 250mL Erlenmeyer flask.
2. Add 100mL PEM buffer to flask.
3. Place stir-bar in flask and set stir plate to high for 10 minutes.

PEMTAX Solution (20mM Taxol):

1. Place a labeled tube on ice.
2. Pipet 1000μL PEM buffer into labeled tube.
3. Pipet 10μL Taxol (2mM stock) into tube.

4. Vortex to mix and place on ice.

Primary Antibody Solution (10µg/mL mouse anti-β-tubulin in blocking solution):

1. Pipet 1000µL blocking solution into labeled tube.
2. Pipet 5µL mouse anti-β-tubulin (2mg/mL stock).
3. Vortex to mix and place on ice.

Secondary Antibody Solution (30µg/mL goat-α-mouse Cy3 in blocking solution):

**ensure that the Cy3 remains covered at all times as it is light sensitive*

1. Pipet 500µL blocking solution into labeled tube.
2. Pipet 10µL goat-α-mouse Cy3 (1.5mg/mL stock).
3. Vortex to mix, cover with foil, and place on ice.

Immunolabeling:

1. Prepare an ice bucket and supply it with PEM buffer, taxol, BSA, primary antibody (anti-β-tubulin), and secondary antibody (Cy3)
2. Prepare 100mL blocking solution.
3. Prepare 1mL PEMTAX solution.
4. Prepare a container of methanol large enough to fit a slide and place at -20°C.
4. Prepare a 1:50 dilution of microtubule stock solution in PEMTAX.
5. Label a poly-L-lysine coated slide and place it in a humid environment (*i.e.* A slide box containing wet paper towels).
6. Pipet 200µL microtubule dilution onto PLL surface. Close the box securely and set at room temperature for 20 minutes.
7. Prepare primary antibody solution.
8. Prepare secondary antibody solution and keep covered on ice.
9. Remove slide from humidity chamber after 20 minutes and rinse in PEM buffer by submerging completely in buffer ten times.

**If staining multiple treatment groups prepare a separate wash for each slide to prevent cross contamination.*

10. Submerge slide ten times in -20°C methanol bath, then three times in PEM buffer.
11. Place slide in blocking solution for 20 minutes at room temperature.

12. Remove slide from blocking solution and gently shake off excess blocking solution.
13. Dry the sides and bottom of the slide on a small stack of paper towels taking care not to touch the PLL surface. Then place it back in the humid chamber.
14. Pipet 1000µL primary antibody solution onto the slide, close the slide box tightly, and set it at room temperature for 30 minutes.
15. Gently shake off excess primary antibody solution and rinse in blocking solution for 10 seconds.
16. Dry the sides and bottom of the slide again on a small stack of paper towels. Then place it back in the humid chamber.
17. Pipet 1000µL secondary antibody solution onto the slide, close the slide box tightly, and set it at room temperature for 2 hours.
18. Gently shake off excess primary antibody solution and rinse in blocking solution for 10 seconds.
19. Rinse in fresh PEM buffer and add a cover slip. The slide is now ready for fluorescent imaging.

Imaging On The Fluorescent Microscope:

1. Reserve microscope ahead of time.
2. Log into sign-in sheet and turn on fluorescent lamp and computer.
3. Ensure all shudders are open and set filter to emit 552nm light (usually green), then close the fluorescent light shudder.
4. Open image acquisition software
5. Set the objective to 100X.
6. Ensure scale-bars are present at proper scale, set fluorescence to manual or automatic, depending on the experiment, and adjust other image acquisition parameters to fit experiment.
7. Place one drop of objective oil directly on 100X objective.
8. Route the image to the eyepiece, open the fluorescent light shudder, and obtain the desired image.
9. Route the image to the computer and acquire the image using the software.
10. Repeat 7 through 10 until finished, then save the images, turn off the lamp and computer, and sign-out.

Microtubule Dissociation (Aggregate Formation) **Experiment:**

1. Prepare an ice bucket and supply it with PEM buffer, GTP, Taxol, tubulin, EB1, and $p150^{glued}$.
2. Place an open microcentrifuge tube that has been labeled microtubule stock solution in the ice bath.
3. Pipet 160µL PEM buffer into the microcentrifuge tube.
4. Pipet 4µL GTP (10mM stock solution) into the microcentrifuge tube.
5. Pipet 4µL Taxol (2mM stock solution) into the microcentrifuge tube by removing the tube from the ice bath and holding it up to a light background during pipeting. This is to ensure the delivery of such a small volume. An oily streak should be ejected from the pipet tip.
6. Cap the tube and vortex to mix.
7. Place the tube back in the ice bath and add 6µL tubulin (10mg/mL).
8. Pipet mix with 200µL pipet.
9. Place the tube in a 37°C incubator for 30 minutes.
10. Pipet 490µL PEM buffer into tube labeled as control.
11. Pipet 450µL PEM buffer into tube labeled as treatment.
12. Pipet 20µL EB1 into treatment group tube.
13. Pipet 20µL $p150^{glued}$ into treatment group tube and pipet mix.
14. After 30 minutes place all three tubes in the ice bath and move to the scope.
15. Begin timer upon adding 10µL microtubule stock solution to each dilution tube.
16. At the desired time points flick the dilution tube gently to mix the solution and pipet 10µL onto a poly-L-lysine coated microscope slide.
17. Add a cover slip and image each time point.

Surface Contrast (SAM Validation) **Experiment:**

1. Obtain 2 anti-GST functionalized surfaces.
2. Rinse in PBS.
3. Pipet 10µL GST-CLIP-170-H2 onto one surface and leave at room temperature for 20 minutes.
4. Rinse in PBS.

5. Pipet 10μL microtubule stock solution onto both chips and set at room temperature for 20 minutes.
6. Prepare Block solution
7. Prepare primary antibody solution
8. Prepare secondary antibody solution
9. Place both chips in block solution at room temperature for 20 minutes.
10. Place both chips in primary antibody solution for 30 minutes.
11. Rinse both chips in block solution.
12. Place both chips in secondary antibody solution for 1 hour.
13. Rinse in PBS. Chips are now ready for imaging.

Preparing SAM Functionalized Gold Surfaces:

1. Obtain a silica wafer.
2. Coat with a thin layer of chromium using physical vapor deposition.
3. Deposit a ~100nm layer of gold using physical vapor deposition.
4. Clean the surface with Piranha solution (1:3 30% hydrogen peroxide: 95% sulfuric acid). This will partially oxidize the gold to Au+.
5. Rinse the chip in water.
6. Submerge chip in 1mM mercaptohexadonic acid in 10% ethanol for 24 hours.

Preparing BPT Solution:

1. Pipet 10mL PEM80 into centrifuge tube.
2. Add 100mg BSA to tube.
3. Pipet 100μL Taxol into tube and vortex thoroughly to mix, then set on ice.

Nucleating Microtubules From Surface Using Regular Tubulin:

1. Remove a chip from the MHA solution and rinse it with purified water.
2. Place the chip on a clean surface approximately 10 inches from the lab bench air line and turn the air stream on low flow to air dry the chip.
3. Prepare a humid environment by placing wet paper towels in a slide box.

4. Submerge chip in 1mg/mL anti-GST + EDC in PEM buffer inside the slide box, close the lid tightly, and set at room temperature for 3-4 hours.
5. Rinse chip in 2%BSA in PEM buffer for 20 minutes at room temperature.
6. Inside the slide box pipet enough 0.05mg/mL GST-γ-tubulin to cover the entire surface, close the lid tightly, and set for 30 minutes at room temperature.
7. Rinse chip in 2%BSA in PEM buffer for 5 minutes at room temperature.
8. Prepare an ice bucket and supply it with PEM buffer, GTP, Taxol, and tubulin.
9. Place an open microcentrifuge tube that has been labeled into the ice bath.
10. Pipet 160μL PEM buffer into the microcentrifuge tube.
11. Pipet 4μL GTP (10mM stock solution) into the microcentrifuge tube.
12. Pipet 4μL Taxol (2mM stock solution) into the microcentrifuge tube by removing the tube from the ice bath and holding it up to a light background during pipeting. This is to ensure the delivery of such a small volume. An oily streak should be ejected from the pipet tip.
13. Cap the tube and vortex to mix.
14. Place the tube back in the ice bath and add 6μL tubulin (10mg/mL).
15. Pipet mix with 200μL pipet.
16. Carefully place the chip in the tube making sure not to touch the surface.
17. Place the tube in a 37°C incubator for 30 minutes.
18. Prepare a bath of -20°C methanol large enough to accommodate a microscope slide.
19. Prepare blocking solution and keep on ice.
20. Prepare regular primary antibody solution and keep on ice.
21. Prepare regular secondary antibody solution and keep covered on ice.
22. Prepare BPT solution and keep on ice.
23. Pipet 1mL BPT into tube labeled primary antibody in BPT solution.

24. Pipet 5μL primary antibody into primary antibody in BPT tube and keep on ice.
25. Pipet 500μL BPT into tube labeled secondary antibody in BPT solution.
26. Pipet 10μL secondary antibody into secondary antibody in BPT tube and keep covered on ice.
27. Remove the tube containing the chip from the incubator and pipet 10μL of the microtubule solution onto a poly-L-lysine coated microscope slide labeled control.
28. Set the slide at room temperature for 10 minutes.
29. Carefully remove the chip from the tube and place into BPT rinse for 30 minutes at room temperature.
30. Submerge slide ten times in -20°C methanol bath, then three times in PEM buffer.
31. Place slide in blocking solution for 20 minutes at room temperature.
32. Submerge the chip in primary antibody in BPT solution for 30 minutes.
33. Remove slide from blocking solution and gently shake off excess blocking solution.
34. Dry the sides and bottom of the slide on a small stack of paper towels taking care not to touch the PLL surface. Then place it back in the humid chamber.
35. Pipet 1000μL primary antibody solution onto the slide, close the slide box tightly, and set it at room temperature for 30 minutes.
36. Rinse the chip in BPT.
37. Place the chip in secondary antibody in BPT solution for 2 hours at room temperature.
38. Gently shake excess primary antibody solution off the slide and rinse it in blocking solution for 10 seconds.
39. Dry the sides and bottom of the slide again on a small stack of paper towels. Then place it back in the humid chamber.
40. Pipet 1000μL secondary antibody solution onto the slide, close the slide box tightly, and set it at room temperature for 2 hours.
41. Gently shake excess secondary antibody solution off the slide and rinse it in blocking solution for 10 seconds.

42. Rinse the slide in fresh PEM buffer and add a cover slip.
43. Place the chip in BPT solution. The functionalized chip and control slide are now ready to be imaged with a fluorescent microscope.

Capturing Microtubules Onto Surface Using Regular Tubulin:

1. Remove a chip from the MHA solution and rinse it with purified water.
2. Place the chip on a clean surface approximately 10 inches from the lab bench air line and turn the air stream on low flow to air dry the chip.
3. Prepare a humid environment by placing wet paper towels in a slide box.
4. Submerge chip in 1mg/mL anti-GST + EDC in PEM buffer inside the slide box, close the lid tightly, and set at room temperature for 3-4 hours.
5. Rinse chip in 2%BSA in PEM buffer for 20 minutes at room temperature.
6. Inside the slide box pipet enough 5.91mg/mL GST-$p150^{glued}$ to cover the entire surface, close the lid tightly, and set for 30 minutes at room temperature.
7. Prepare an ice bucket and supply it with PEM buffer, GTP, Taxol, and tubulin.
8. Place an open microcentrifuge tube that has been labeled into the ice bath.
9. Pipet 140µL PEM buffer into the microcentrifuge tube.
10. Pipet 4µL GTP (10mM stock solution) into the microcentrifuge tube.
11. Pipet 4µL Taxol (2mM stock solution) into the microcentrifuge tube by removing the tube from the ice bath and holding it up to a light background during pipeting. This is to ensure the delivery of such a small volume. An oily streak should be ejected from the pipet tip.
12. Cap the tube and vortex to mix.
13. Place the tube back in the ice bath and add 6µL tubulin (10mg/mL).
14. Pipet mix with 200µL pipet.
15. Place the tube in a 37°C incubator for 15 minutes.

16. Pipet 10µL 1.30mg/mL EB1 and 10µL 0.1mg/mL HIS-CLIP-170-H2 into the tube.
17. Pipet mix with 200µL pipet and place back in the incubator for 15 minutes.
18. Prepare a bath of -20°C methanol large enough to accommodate a microscope slide.
19. Prepare blocking solution and keep on ice.
20. Prepare regular primary antibody solution and keep on ice.
21. Prepare regular secondary antibody solution and keep covered on ice.
22. Prepare BPT solution and keep on ice.
23. Pipet 1mL BPT into tube labeled primary antibody in BPT solution.
24. Pipet 5µL primary antibody into primary antibody in BPT tube and keep on ice.
25. Pipet 500µL BPT into tube labeled secondary antibody in BPT solution.
26. Pipet 10µL secondary antibody into secondary antibody in BPT tube and keep covered on ice.
27. Remove the tube containing the microtubule stock solution from the incubator and pipet 10µL of the microtubule solution onto a poly-L-lysine coated microscope slide labeled control.
28. Set the slide at room temperature for 10 minutes.
29. Submerge slide ten times in -20°C methanol bath, then three times in PEM buffer.
30. Place slide in blocking solution for 20 minutes at room temperature.
31. Rinse the chip in 2%BSA in PEM buffer for 5 minutes at room temperature.
32. Pipet 288µL BPT into a tube labeled microtubule dilution.
33. Pipet 12µL microtubule stock solution into tube labeled microtubule dilution and pipet mix.
34. Carefully place the chip in the microtubule dilution tube making sure not to touch the surface, close the lid, and place at room temperature for 30 minutes.
35. Place the chip into BPT rinse for 30 minutes at room temperature.

36. Submerge the chip in primary antibody in BPT solution for 30 minutes.
37. Remove slide from blocking solution and gently shake off excess blocking solution.
38. Dry the sides and bottom of the slide on a small stack of paper towels taking care not to touch the PLL surface. Then place it back in the humid chamber.
39. Pipet 1000µL primary antibody solution onto the slide, close the slide box tightly, and set it at room temperature for 30 minutes.
40. Rinse the chip in BPT.
41. Place the chip in secondary antibody in BPT solution for 2 hours at room temperature.
42. Gently shake excess primary antibody solution off the slide and rinse it in blocking solution for 10 seconds.
43. Dry the sides and bottom of the slide again on a small stack of paper towels. Then place it back in the humid chamber.
44. Pipet 1000µL secondary antibody solution onto the slide, close the slide box tightly, and set it at room temperature for 2 hours.
45. Gently shake excess secondary antibody solution off the slide and rinse it in blocking solution for 10 seconds.
46. Rinse the slide in fresh PEM buffer and add a cover slip.
47. Place the chip in BPT solution. The functionalized chip and control slide are now ready to be imaged with a fluorescent microscope.

References - IX

1. Valiron O, Caudron N, Job D. Microtubule Dynamics. Cell Mol Life Sci. 2001 Dec;58(14):2069-84. Review.

2. Behrens S. Rahn K. Habicht W. Nanoscale Particle Arrays Induced by Highly ordered Protein Assemblies. 2002 Advanced Materials (4)No.22, Nov 18

3. Kirsch R. Mertig M. Pompe R. Wahl R. Three-Dimensional Metallization of Microtubules. Thin Solid Films. 1997 Feb 26.

4. Mertig M. Kirsch R. Pompe W. Biomolecular Approach to Nanotube Fabrication. 1998 Applied Physics and Materials Science and Processing 66, S723-S727 October

5. Yang Y. Deymier P. Wang L. Guzman R. Hoying J Nucleation and Growth of Microtubules from Gamma-Tubulin-Functionalized Gold Surfaces. Biotechnol. Prog. 2006, 22, 303-312

6. Aldaz H, Rice LM, Stearns T, Agard DA. Insights into microtubule nucleation from the crystal structure of human gamma-tubulin. Nature. 2005 May 26;435(7041):523-7.

7. Schilstra M. Bayley P. Martin S. The Effect of Solution Composition on Microtubule Dynamic Instability. Biochem. J. (1991) 277, 839-847

8. R. A. Walker, N. K. Pryer, and E. D. Salmon . Dilution of Individual Microtubules Observed in Real Time In Vitro : Evidence That Cap Size Is Small and Independent of Elongation Rate. The Journal of Cell Biology, Volume 114, Number 1, July 1991 73-81.

9. Schiff P. Fant B. Horwitz J. Promotion of Microtubule Assemble in vitro by Taxol. Nature 1979 (vol.277) Feb 22

10. Gaskin F. Cantor C. Turbidimetric Studies of the in Vitro Assembly and Disassembly of Porcine Neurotubules. J. Mol. Biol. (1974) 89, 737-758 Apr 4

11. Rao S. He L. Chakravarty S. Characterization of the Taxol Binding Site on the Microtubule. 1999 J Biol. Chem. Vol. 274 (53), Dec. 31

12. Job D, Valiron O, Oakley B. Microtubule Nucleation. Curr Opin Cell Biol. 2003 Feb;15(1):111-7. Review.

13. Leguy R, Melki R, Pantaloni D, Carlier MF. Monomeric Gamma-Tubulin Nucleates Microtubules. J Biol Chem. 2000 Jul 21;275(29):21975-80.

14. Moritz M, Braunfeld MB, Guenebaut V, Heuser J, Agard DA. Structure of the Gamma-Tubulin Ring Complex: A Template for Microtubule Nucleation. Nat Cell Biol. 2000 Jun;2(6):365-70.

15. Wiese C, Zheng Y. A New Function for the Gamma-Tubulin Ring Complex as a Microtubule Minus-End Cap. Nat Cell Biol. 2000 Jun;2(6):358-64.

16. Schiebel E. Gamma Tubulin Complexes: Binding to the Centrosome, Regulation, and Microtubule Nucleation. Current Opinion in Cell Biology 2000, 12:113-118.

17. Hall D. Minton A. Turbidity as a probe of tubulin polymerization kinetics: A theoretical and experimental re-examination. Analytical Biochemistry, Vol. 345, Issue 2, 15 October 2005.

18. Mimori-Kiyosue Y, Tsukita S. "Search-and-capture" of Microtubules Through Plus-End-Binding Proteins (+TIPs). J Biochem (Tokyo). 2003 Sep;134(3):321-6. Review.

19. Ligon LA, Shelly SS, Tokito MK, Holzbaur EL. Microtubule binding proteins CLIP-170, EB1, and p150Glued form distinct plus-end complexes. FEBS Lett. 2006 Feb 20;580(5):1327-32. Epub 2006 Jan 26.

20. Scheel J, Pierre P, Rickard JE, Diamantopoulos GS, Valetti C, van der Goot FG, Haner M, Aebi U, Kreis TE. Purification and analysis of authentic CLIP-170 and recombinant fragments.
J Biol Chem. 1999 Sep 3;274(36):25883-91.

21. Pierre P, Pepperkok R, Kreis TE. Molecular characterization of two functional domains of CLIP-170 in vivo. J Cell Sci. 1994 Jul;107 (Pt 7):1909-20.

22. Hayashi I, Wilde A, Mal TK, Ikura M. Structural Basis for the Activation of Microtubule Assembly by the EB1 and p150Glued Complex. Mol Cell. 2005 Aug 19;19(4):449-60.

23. Perez F, Diamantopoulos GS, Stalder R, Kreis TE. CLIP-170 Highlights Growing Microtubule Ends *in vivo*. Cell. 1999 Feb 19;96(4):517-27.

24. Folker ES, Baker BM, Goodson HV. Interactions between CLIP-170, tubulin, and microtubules: implications for the mechanism of Clip-170 plus-end tracking behavior.
Mol Biol Cell. 2005 Nov;16(11):5373-84. Epub 2005 Aug 24.

25. Arnal I, Heichette C, Diamantopoulos GS, Chretien D. CLIP-170/Tubulin-Curved Oligomers Coassemble at Microtubule Ends and Promote Rescues. Curr Biol. 2004 Dec 14;14(23):2086-95.

26. Tanenbaum ME, Galjart N, van Vugt MA, Medema RH. CLIP-170 facilitates the formation of kinetochore-microtubule attachments. EMBO J. 2006 Jan 11;25(1):45-57. Epub 2005 Dec 15.

27. Goodson H. Skube S. Stalder R. Valetti C. Kreis T. Morrison E. Schroer T. Clip-170 Interacts With Dynactin Complex and the APC-Binding Protein EB1 by Different Mechanisms. Cell Motility and the Cytoskeleton. 55:156-173, 2003

28. Watson P. Stephens D. Microtubule Plus-End Loading of P150glued is Mediated by EB1 and Clip-170 But Is Not Required for Intracellular Membrane Traffic In Mammalian Cells. Journal of Cell Science. 2006, 119 (13)

29. Komarova Y, Lansbergen G, Galjart N, Grosveld F, Borisy GG,

Akhmanova A. EB1 and EB3 Control CLIP Dissociation From the Ends of Growing Microtubules. Mol Biol Cell. 2005 Nov;16(11):5334-45. Epub 2005 Sep 7.

30. Bu W, Su LK. Characterization of Functional Domains of Human EB1 Family Proteins. J Biol Chem. 2003 Dec 12;278(50):49721-31. Epub 2003 Sep 26.

31. Askham J. Vaughan K. Goodson H. Morrison E. Evidence That an Interaction Between EB1 and p150glued Is Required for the Formation and Maintenance of a Radial Microtubule Array Anchored at the Centrosome. Molecular Biology of the Cell, Vol.13, 3627-3645, October 2002.

32. Tirnauer J. Canman J. Salmon E.D. Mitchison T. EB1 Targets to Kinetochores with Attached, Polymerizing Microtubules. Molecular Biology of the Cell, Vol.13, 4308-4316, December 2002.

33. Ligon L. Shelly S. Tokito M. Holzbaur E. The Microtubule Plus-End Proteins EB1 and Dynactin Have Differential Effects on Microtubule Polymerization. Molecular Biology of the Cell, Vol. 14, 1405-1417, April 2003.

34. Strickland L. Wen Y. Gundersen G. Burgess D. Interaction Between EB1 and p150glued Is Required for Anaphase Astral Microtubule Elongation and Stimulation of Cytokinesis. Current Biology, Vol.15, 2249-2255, December 20, 2005.

35. Wang, L., Yang, Y., McLaughlin, H., Jongewaard, I., Hoying, J., Deymier, P. A. and Guzmán, R., Molecular Assembly for the Functionalization of a Gold Surface with a Gamma-Tubulin as a Biospecific Linker, *Biochimica etBiophysica Acta*.